New vaccines against pneumonia:

Investigating protein-specific immune responses to *Streptococcus pneumoniae* using Experimental Human Pneumococcal Carriage

Thesis submitted in accordance with the requirements of
the University of Liverpool for the degree of Doctor in Philosophy by

Jessica Taripre Owugha

May 2017

“Side by side in the lung

All air is renewed

In constant opposition”

Eduardo Cury (verse) b. 1963

Marco Ulgheri (artwork) b. 1970

Title page image: ‘The discovery of pulmonary circulation’ bronze plaque as part of the permanent exhibition entitled ‘The history of medicine’, Santa Catarina Hospital, São Paulo, Brasil. Eighth of 24 panels following the progression of medicine along the hospital walls: from traditional religious through to contemporary genetic approaches, this plaque highlights the age-old history of lung disease. The text alongside describes the seeming juxtapositioning of gaseous exchange. For this thesis, the mention of opposition in the descriptive text punctuates the importance of investigating potentially beneficial immune responses at the site of infection, where opposition between pneumococcal infection and homeostatic function occurs, side by side. Photograph credit Jessica T. Owugha, 2014.

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“Thought has always worked by opposition”

Hélène Cixous b. 1937

Hélène Cixous is a Jewish-Algerian-French poet and philosopher: her writings are born out of the juxtaposed nature of her genealogy and life story. The quote above reflects these themes of contradiction, and relates to this thesis in reinforcing the message of opposing pathogen and homeostatic functions in the lung, and the need to research, that is to think about, immune responses at this site of infection in pneumonia vaccine discovery efforts.

Declaration

This thesis is the result of the original work of the author. In some instances, studies were conducted in collaboration with internal colleagues and external collaborators; individual roles are outlined overleaf. The laboratory experiments presented in this thesis were carried out predominantly at the Liverpool School of Tropical Medicine, United Kingdom, but in part at Instituto Butantan, Brasil. The contents of this thesis have not been presented, nor are currently being presented, wholly or in part, for any other degree or qualification.

Jessica Taripre Owugha

May 2017

Collaborators of/ contributors to work presented in this thesis

Activity	**Responsibility**
Recruitment, sample taking, inoculation, microbiology	Angela D. Wright, Andrea M. Collins, Carole Hancock, Elena Mitsi, Jenna F. Gritzfeld, Stephen B. Gordon (Liverpool School of Tropical Medicine)
PspA protein expression and purification	Adriana T. Moreno (Instituto Butantan)
Epitope identification	Joshua Blight (Jenner Institute)
Statistical support	Duolao Wang & Brian Faragher (Liverpool School of Tropical Medicine)

Abstract

New vaccines against pneumonia: investigating protein-specific immune responses to *Streptococcus pneumoniae* using Experimental Human Pneumococcal Carriage

J. T. Owugha

Infections caused by *Streptococcus pneumoniae* are a major cause of morbidity and mortality globally: *S. pneumoniae* is the primary bacterial agent of pneumonia, the leading cause of death in children under 5 years of age and a major cause of invasive disease in the elderly. Currently licensed vaccination strategies target pneumococcal capsular polysaccharides of which there are 94 known types (serotypes). Antibodies against vaccine serotypes effectively protect against invasive pneumococcal disease, but less so against mucosal infections including pneumonia. New vaccine development efforts aim to overcome these hurdles by targeting conserved and immunogenic pneumococcal proteins. The Pneumococcal Surface Protein A (PspA) is a leading candidate due to its expression across all known clinically relevant pneumococcal strains, and ability to protect against infection across serotypes. However, PspA sequence heterogeneity necessitates identification of cross-protective protein constructs. In this doctoral research project, I investigated pneumococcal protein-specific humoral and $CD4^+$ T-lymphocyte immune responses to *S. pneumoniae* utilising the platform of Experimental Human Pneumococcal Carriage (EHPC). With an emphasis on PspA, immune responses to nasopharyngeal carriage - the prerequisite to pneumococcal disease and proxy for infection - were evaluated.

I present the first evidence of $CD4^+$ T-cell responses specific to an individual pneumococcal purified protein in the blood of healthy adults both before and after carriage. PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* in bronchoalveolar lavage of healthy adults after EHPC were detected at lower levels than in blood, indicating non-immunodominance of PspA as a T-cell antigen in the lung, and compartmentalisation of immune response. I also describe from preliminary data, a potential role of PspA in protection from pneumococcal carriage re-acquisition, and optimise an assay for the identification of linear pneumococcal protein epitopes with potential for inclusion in a multiple epitope protein.

Unveiling protein-specific immune responses to *S. pneumoniae* using controlled and reproducible EHPC, may aid in our understanding of immunity towards selection of protein vaccine candidates for protection against human pneumococcal infection and disease.

List of publications

Peer-reviewed papers

PspA immunity in adulthood: IgG responses to PspA proteins, fragments and linear epitopes in adults young and old

Owugha JT*, Vadesilho CFM*, Moreno AT, Gritzfeld JF, Mitsi E, Wright AD, Collins AM, Walsh S, Briles DE, Gordon SB, Miyaji EN[#], Ferreira DM[#] (manuscript in preparation)

Human Pneumococcal surface protein A-specific $CD4^+$ T-cell responses to Experimental Human Pneumococcal Carriage

Owugha JT, Mitsi E, Gritzfeld J, Collins A, Jambo KC., Miyaji EN, Gordon SB, Ferreira DM* (manuscript in preparation)

Agglutination by anti-capsular polysaccharide antibody is associated with protection against experimental human pneumococcal carriage

Mitsi E, Roche AM, Reiné J, Zangari T, **Owugha JT**, Pennington SH, Gritzfeld JF, Wright AD, Collins AM, van Selm S, de Jonge MI, Gordon SB, Weiser JN, Ferreira DM. Mucosal Immunol. 2016

Polysaccharide-Specific Memory B Cells Predict Protection against Experimental Human Pneumococcal Carriage

Pennington SH, Pojar S, Mitsi E, Gritzfeld JF, Nikolaou E, Solórzano C, **Owugha JT**, Masood Q, Gordon MA, Wright AD, Collins AM, Miyaji EN, Gordon SB, Ferreira DM. Am J Respir Crit Care Med. 2016

Single-use and Conventional Bronchoscopes for Broncho alveolar Lavage (BAL) in Research: a comparative study (NCT 02515591)

Zaidi S*, Collins AM*, Mitsi E, Davies K, Wright A, **Owugha JT**, Reiné J, Fitzgerald R, Ganguli A, Gordon SB, Ferreira DM, Rylance J. BMC Pulm Med. 2017

Accepted conference abstracts

British Society of Immunology Summer School 2014

New protein vaccine against pneumococcal pneumonia using experimentally acquired immunity

Owugha JT, Vadesilho CFM, Pennington SH, Preciado-Llanes L, Jambo KC, Banyard A, Gordon SB, Ferreira DM

EuroPneumo 2015

New protein vaccines against pneumococcal pneumonia using experimentally induced immunity

Owugha JT, Wright A, Collins A, Vadesilho CFM, Miyaji EN, Jambo KC, Gordon SB, Ferreira DM

International Symposium of Pneumococci and Pneumococcal Disease (ISPPD) 2016

Human pneumococcal carriage increases cross-reactivity of anti-PspA IgG with heterologous family PspAs, but protection against re-acquisition is restricted within PspA family types

Owugha JT, Vadesilho CFM, Moreno AM, Walsh S, Briles DE, Gordon SB, Miyaji EN, Ferreira DM

Acknowledgements

First and foremost, I acknowledge my Lord and Saviour Jesus Christ - only by your grace, this project and I are where we are today.

I thank my primary and secondary supervisors for providing the opportunity to conduct this research within the Respiratory Infection Group (RIG) – your attentive academic guidance and pastoral support has been invaluable. I am grateful to The RIG, both past and present – it has been great working with you all and to be part of a clinical trial team. I especially acknowledge the study volunteers who made these studies possible.

To all at the LSTM that have journeyed with me since the start of the PhD: from students to visiting scientists, postdoctoral researchers to professors and all non-academic staff. From the odd kind word to hours of Flow Cytometry training - thank you. To the wider Department of Clinical Sciences and LSTM committees for funding my studentship and awards throughout my program. Reciprocally so for the opportunities offered to contribute to the broader fabric of the institution's policies and strategic direction. All endeavours have strengthened my research, and me as a researcher, for which I am grateful.

To my friends old and new, far and wide. From across Africa to Annecy, Lausanne to London, Madrid to Manchester, São Paulo to Scotland, here in Liverpool and everywhere and everyone in between. Thank you for all the laughter, retreat locations, standing alongside me and cheering me on.

And last but not least, to my family – for your endless patience, lending of ears, tireless encouragement, and for all that there are no words to scribe. This degree is ours.

"There is no 'I' in PhD!" - J. T. Owugha, 2017.

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List of abbreviations

aa	amino acid
BAL	bronchoalveolar lavage
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
°C	degrees Celsius
CD4	cluster of differentiation 4
CI	confidence interval
CFSE	carboxyfluorescein succinimidyl ester
CT	cycle threshold
EHPC	Experimental Human Pneumococcal Carriage
F (F2, F4, F7)	Fragment
FC	Fold Change
FI	fluorescence intensity
FITC	fluorescein isothiocyanate
g	gravitational force
HDC	highly diverse control
Ig	immunoglobulin
IPD	invasive pneumococcal disease
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilo Dalton
LRTI	lower respiratory tract infection
µg	microgram
mM	mili Mole
M	Mole
mL	millilitre
µL	microliter
NBT	nitro blue tetrazolium
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PC	positive control
PCV	Polysaccharide conjugate vaccine
PF	precursor frequency

pH	potential of hydrogen
PHA	*Phaseolus vulgaris* phytohaemagglutinin
PspA	Pneumococcal surface protein A
qPCR	quantitative polymerase chain reaction
rpm	revolutions per minute
RPMI	Roswell park memorial institute medium
SD	standard deviation
SDS	sodium dodecyl sulphate
S. pneumoniae	*Streptococcus pneumoniae*
TBST	Tris buffered saline plus tween
T-cell	thymus lymphocyte cell
Th-(1/2/17) cell	T-helper (1/2/17) cell
THY	Todd Hewitt medium plus yeast extract
UGPI	upper generation proliferation index

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CHAPTER 1

General introduction

1 General introduction

1.1 Pneumococcal disease epidemiology

Infections caused by *Streptococcus pneumoniae* are a major cause of morbidity and mortality globally. With an estimated annual case fatality of 1.6 million, children in developing nations bear a disproportionate level of the burden (UNICEF, 2006). Childhood pneumonia of which *S. pneumoniae* is the primary bacterial aetiological agent, is estimated to cause 150 million episodes per year in developing nations alone, accounting for over 95% of the global burden. Two million children die, 2000 times more than in industrialised nations (Rudan *et al*., 2004).

In Europe and the U.S.A, *S. pneumoniae* infections are the most common cause of bacterial community acquired pneumonia (CAP) in adults (Musher and Thorner, 2014). 2013/14 surveillance data from England and Wales place CAP and non-invasive disease at an incidence rate of 20.6 per 100,000 adults, and invasive pneumococcal disease (IPD) at 6.85 per 100,000 adults. The burden of IPD increases to 20.6 per 100,000 adults in the over 65 age stratification (Chalmers *et al*., 2016).

Despite effective vaccine implementation programmes, pneumonia remains a global health priority as the cause of a large burden of childhood deaths (together with diarrhoea). In 2015 alone, over 920,000 children under the age of 5 were killed by pneumonia, most victims being under 2 years of age (WHO, 2016), where the majority of fatalities occurred in sub-Saharan Africa and South Asia (Figure 1.1). This prompted the WHO and United Nations Children's Fund (UNICEF) to launch the integrated Global Action Plan for the Prevention and Control of Pneumonia and Diarrhoea (GAPPD), which sets out proposals for ending preventable childhood deaths due to pneumonia by the year 2025. One such initiative is the Global Vaccine Action Plan (WHO, 2013): the program highlights strategies for the prevention of childhood disease through vaccination, and its aims include development of next generation vaccines and their introduction.

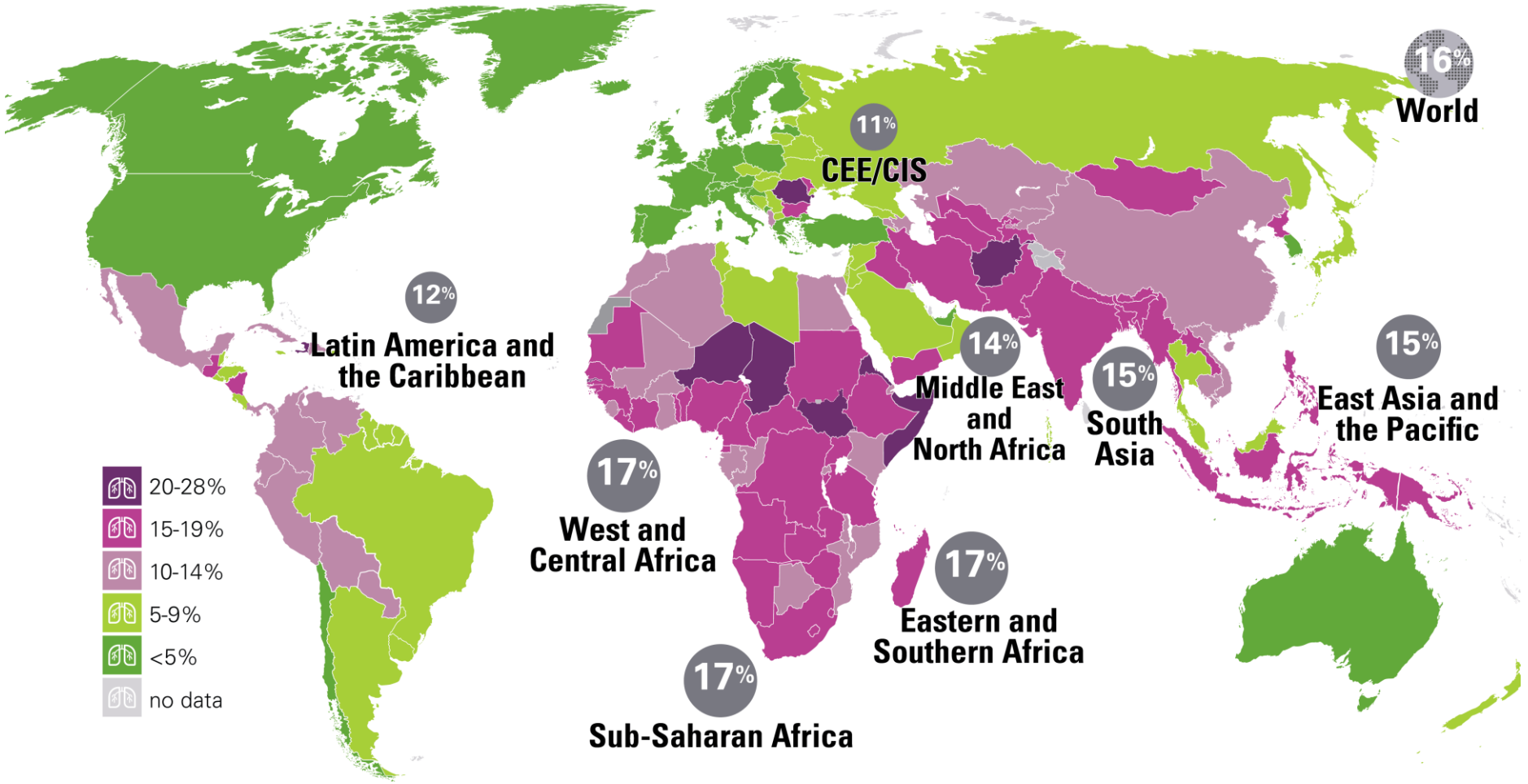


Figure 1.1 Pneumonia-attributable deaths in children under 5 years of age. Estimated percentage of childhood deaths of which pneumonia was the cause in 2015. CEE / CIS – Central and Eastern Europe / Commonwealth of Independent States.

Source: WHO and Maternal and Child Epidemiology Estimation Group (MCEE).

1.2 At risk groups

Age is the primary risk factor in susceptibility to *S. pneumoniae* infection. People at the extremes of age - under 5s (particularly under 2s) and over 65s - are at an increased risk of pneumococcal infection (Public Health England, 2013).

Certain medical conditions also predispose one to pneumococcal infection (Torres *et al*., 2015, Rozenbaum *et al*., 2012). These may fall under the banner of chronic conditions such as:

- respiratory disease inc. chronic obstructive pulmonary disease (COPD), bronchitis, emphysema
- heart disease inc. ischemic and congenital, hypertension with cardiac complications
- kidney disease inc. stages 4 and 5, individuals on dialysis
- liver disease inc. cirrhosis, hepatitis
- diabetes mellitus

or those which alter immunity (Ricerca *et al*., 2009, Jain *et al*., 2015a, Jain *et al*., 2015b) including:

- HIV infection
- respiratory viral infection
- chemotherapy patients
- asplenia or splenic dysfunction inc. sickle cell disease, coeliac syndrome
- malnutrition and under-nourishment

Further still, environmental factors thought to contribute to pneumococcal infection susceptibility (Greenberg *et al*., 2006, Reisman *et al*., 2014) include:

- smoke inc. indoor air pollution from biomass fuel, smoking;
- crowded living conditions

However, a recent cluster randomised control trial of 10,750 children comparing pneumonia rates associated with traditional indoor cooking methods and a cleaner burning biomass-

fuelled cook stove intervention, revealed no difference in risk of pneumonia in a rural region of Malawi, South Eastern Africa (Mortimer *et al*., 2017).

Taken together, it is conceivable as to why the majority of the pneumococcal disease burden falls onto developing nations due to the increasing burden of non-communicable chronic diseases, geographical co-distribution of haemoglobinopathies, increasing communicable infections, in addition to long-lasting struggles with undernutrition and poor living conditions, pneumococcal disease truly is a global burden.

1.3 Clinical presentations

1.3.1 Pneumococcal carriage

Streptococcus pneumoniae is a predominantly human opportunistic pathogen and a constituent of the normal human upper respiratory tract microbiome. It colonises the nasopharynx in a state known as pneumococcal carriage (Bogaert *et al*., 2004): although usually asymptomatic in adults (Dowson, 2004), carriage has been associated with rhinitis in children (Rodrigues *et al*., 2013a). The nasopharynx, especially of colonised children, is the reservoir, and transmission occurs by respiratory droplets on person-to-person contact (Bogaert *et al*., 2004).

Importantly, pneumococcal carriage is a pre-requisite to the broad spectrum of pneumococcal disease (Figure 1.2). Host innate and adaptive immunity affect pneumococcal colonisation density and clearance. Together with differential expression of *S. pneumoniae* virulence factors facilitating immune evasion and tissue breach (Miyaji *et al*., 2013), the range of pneumococcal diseases differ in severity and frequency (Figure 1.3).

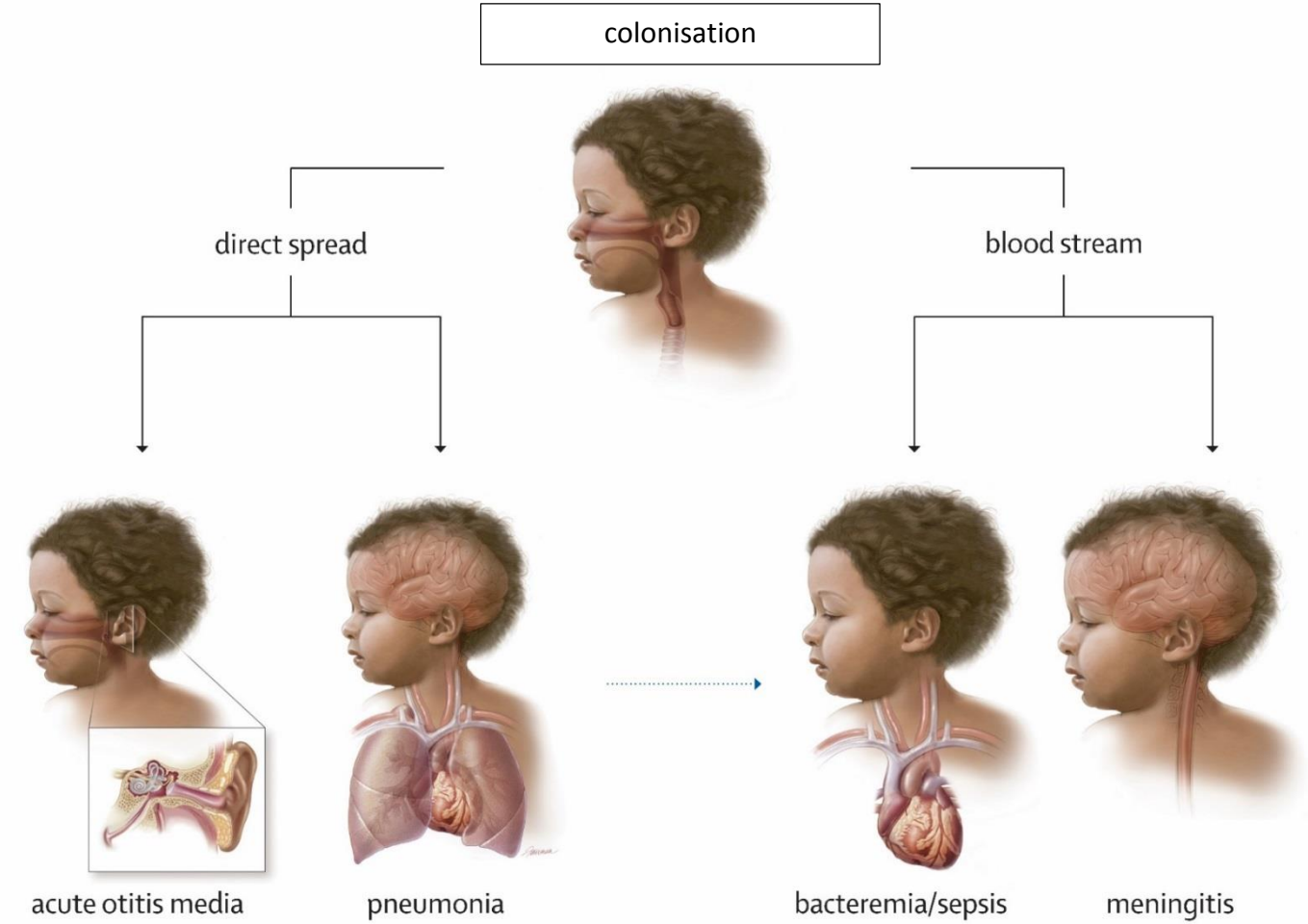


Figure 1.2 Pneumococcal pathogenesis and the spectrum of pneumococcal disease. *Streptococcus pneumoniae* nasopharyngeal colonisation may result in direct spread of pneumococci causing acute otitis media (middle ear infection), or pneumonia (infection of the lung). Pneumococcal translocation into the bloodstream can cause bacteraemia/sepsis (blood and multiple organ infection), or meningitis (blood brain barrier (BBB) breach on systemic infection or CSF leakage on middle ear infection). Figure courtesy of Wouter deSteenhuijsen Piters [Adapted December 2016].

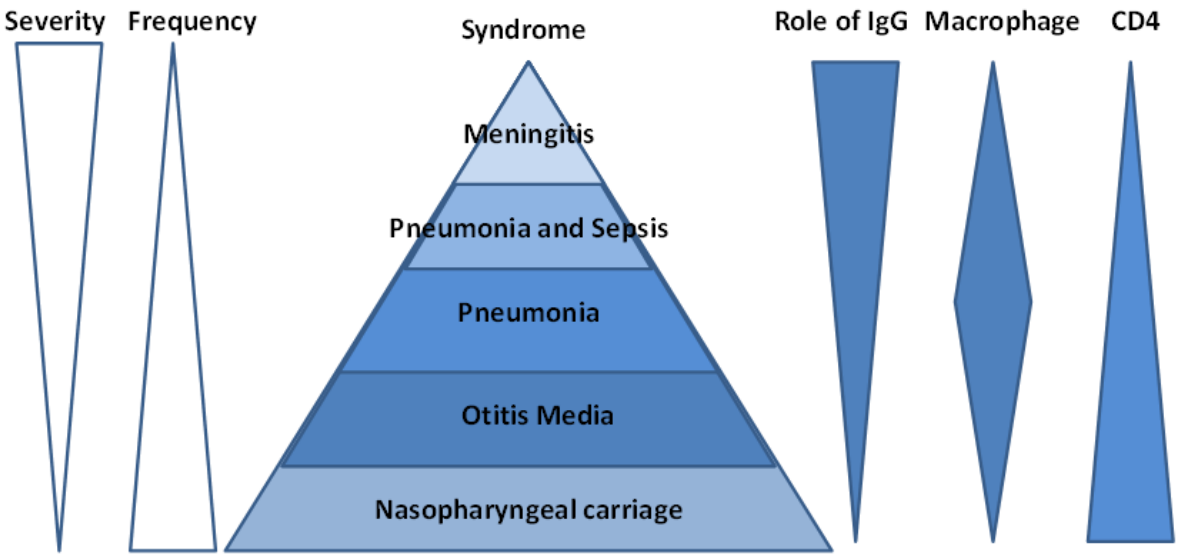


Figure 1.3 Hierarchical chart of pneumococcal syndromes and diseases illustrating their relative associated levels of severity, frequency and immunological defences required to overcome.

1.3.2 Mucosal disease

Mucosal diseases such as otitis media and sinusitis are the most common non-invasive pneumococcal infections. Otitis media (OM) is the infection and inflammation of the middle ear especially common in young children. *S. pneumoniae* traverse the Eustachian tube from the site of nasopharyngeal colonisation to the middle ear where infection can be symptomatic (acute OM) e.g. fever, otalgia, ottorrhoea, or asymptomatic (OM with effusion). Infection of the sinuses, sinusitis, can cause complications such as orbital cellulitis and abscess formation (Chandler, 1970).

1.3.3 Invasive disease

Invasive pneumococcal disease (IPD) includes septicaemia, meningitis and pneumonia. Although less common than mucosal diseases, they are more severe due to the lower immune tolerance capacity of the site of infection, resulting in increased tissue damage (Orihuela *et al*., 2004).

Direct spread of *S. pneumoniae* to the blood (pneumococcaemia) results in symptomatic bacteraemia/septicaemia. The initial hyper-inflammatory phase of infection is followed by a hypo-inflammatory phase: this immune dysregulation increases risk of secondary infections, and can progress to multiple-organ failure (Morton *et al*., 2014). Septicaemia can result in pneumococcal cerebrospinal fluid (CSF) invasion causing meningitis. Subsequent neutrophil CSF influx results in apoptosis and necrosis of brain tissue and resultant brain damage (Weber and Tuomanen, 2007).

1.3.3.1 Pneumonia

Pneumococcal pneumonia is an invasive disease resulting from initial pulmonary infection. However, subsequent bacterial outgrowth results in pulmonary consolidation and infection of the lung alveoli, where breathing becomes laboured and painful due to limited oxygen intake when alveoli are filled with exudate and fluid. Additional clinical presentations of pneumococcal pneumonia (Centre for Disease Control, 2015) include:

- sudden onset of fever, chills or rigors
- pleuritic chest pain
- productive cough with rusty-coloured sputum
- dyspnea, tachypnea, hypoxia
- tachycardia, malaise, weakness
- nausea, vomiting
- headaches

1.4 *Streptococcus pneumoniae* biology

1.4.1 Identification and biochemical features

Streptococcus pneumoniae is a facultative anaerobic, Gram positive bacterium. Colony morphology on defibrinated blood agar appears as alpha-haemolytic, draughtsman colonies (depressed centres due to partial autolysis). Microscopically, pneumococcal morphology is described as a lancet-shaped coccus, often observed as diplococci (pairs). Subculture onto chocolate blood agar for biochemical tests of catalase (negative), optochin (susceptible) and bile solubility (soluble) are indicative of *S. pneumoniae* isolation. Together, these typical morphological features and biochemical tests are the gold standard of *S. pneumoniae* identification. Due to their cost-effectiveness, they are a means of identification worldwide: isolation of a bacterium with aforementioned results from blood, cerebrospinal fluid or other normally sterile body sites is suggestive of pneumococcal infection (CDC, 2015).

Further characterisation of *S. pneumoniae* is based upon tests to identify the predominant antigen, capsular polysaccharide (CPS). With over 94 known serotypes (CPS type), pneumococci can be classified by serotype-specific antibody to CPS (serotyping), as an immunochemically distinct CPS is the defining feature of a serotype, despite their wide antigenic variation. The gold-standard for serotyping is the Quellung reaction: a positive reaction is visualised as a 'swollen' capsule due to the refractive capsule index change on antigen-capsule complex formation. Other serotyping techniques centre around the agglutination reaction, where a positive result is visualised as 'clumping' of bacteria as large antigen-antibody complexes become insoluble and precipitate (including slide, tube and passive agglutination e.g. latex particles).

Genotypic methods of characterisation provide a less subjective method of serotyping with greater discriminatory power, differentiating between strains that may be classed identical by phenotypic assays (Al-Swailem *et al*., 2004), or mixed serotype infection in any clinical sample. Use of genetic serotyping has aided more accurate strain identification and is often employed in surveillance operations monitoring disease burden.

1.4.2 Structure and function: virulence factors

1.4.2.1 Capsular polysaccharide

The capsular polysaccharide (CPS) of *S. pneumoniae* is its major virulence factor and therefore, the primary target for licensed pneumococcal vaccination. The capsule primarily acts to prevent opsonophagocytosis by mediating complement-dependent phagocytosis (Hyams *et al*., 2010a).

Phase variation in *S. pneumoniae* opacity phenotype is associated with pneumococcal pathogenicity: transparent often capsule deficient strains exhibit more invasive potential than their opaque, often capsule rich variants (Ring *et al*., 1998). Reduced capsule expression promotes adherence molecule protrusion and access to host cells, increasing binding to epithelial surfaces to establish colonisation (Talbot *et al*., 1996). However, opacity phase variation is multifactorial and includes differing expression of capsule-associated genes in addition to capsule itself (Chai *et al*., 2017): an example is expression of teichoic acid, where increased expression has been shown to increase nasopharyngeal adherence (Kim and Weiser, 1998).

1.4.2.2 Proteins

Streptococcus pneumoniae expresses numerous virulence proteins, some of the most studied of which are illustrated in Figure 1.4.

Those capable of eliciting protection on immunisation are highlighted in colour, and can be broadly categorised as follows:

- choline-binding proteins e.g. Pneumococcal surface proteins A (PspA) and C (PspC)
- divalent metal-ion-binding lipoproteins e.g. Pneumococcal surface adhesin A (PsaA) which has manganese specificity in an ABC transport system
- LPXTG-anchored proteins e.g. Neuraminidases (NanA)
- enzymes e.g. pneumolysin (Ply), autolysin (LytA, B & C)

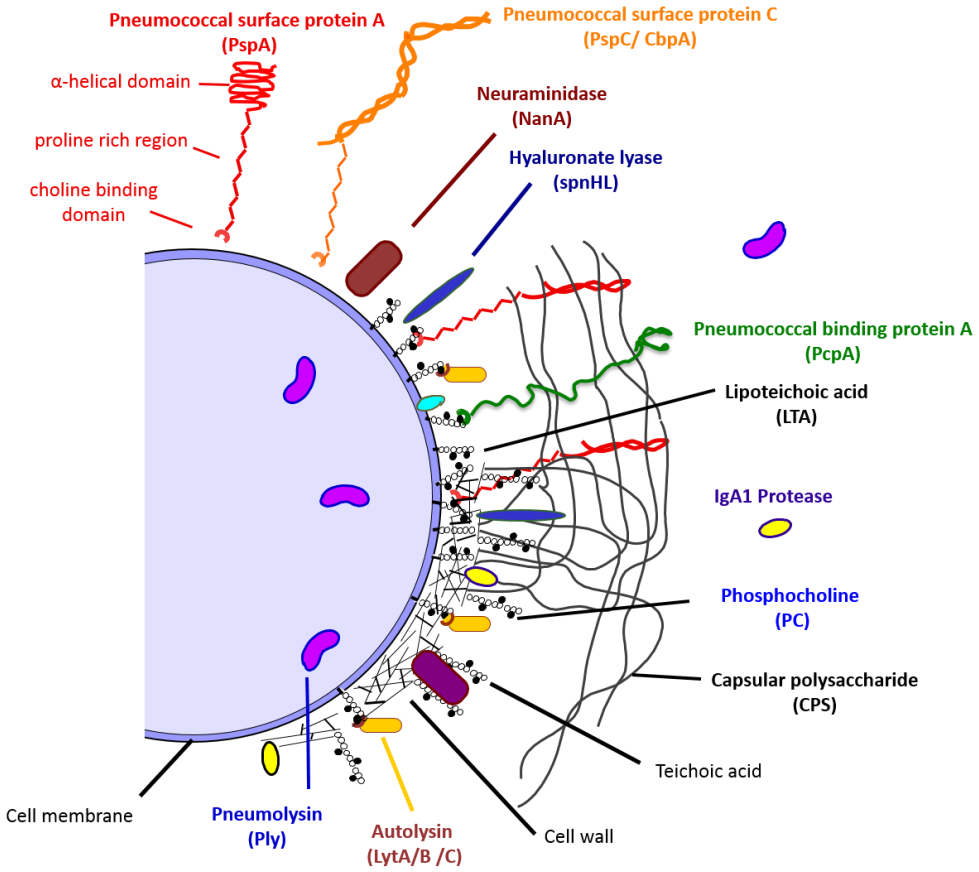


Figure 1.4 *Streptococcus pneumoniae* virulence factors. Well characterised pneumococcal virulence factors with protection-eliciting proteins indicated in colour. Image courtesy of Professor D. Briles [Adapted December 2016].

Table 1.1 lists thirty of the most studied pneumococcal proteins, and summarises their roles in *S. pneumoniae* virulence.

Table 1.1 Well characterised pneumococcal proteins

Antigen	Abbreviation & alternative names	Role in virulence	References
1. Pneumococcal surface protein C	PspC, CbpA, SpsA, Hic	Mediates mucosal adherence, colonisation and invasion Binds host immune factors inc. Factor H, C3, sIgA	Balachandran *et al.*, 2002 Rosenow *et al.*, 1997
2.Pneumococcal surface protein A	PspA	Inhibits complement deposition Prevents apolactoferrin binding and killing	Ren *et al.*, 2004
3. Pneumolysin	Ply	Promotes host cell death by thiol-activated cytolysis on binding cholesterol in cell membranes	Mitchell and Dalziel, 2014 Steinfort *et al.*, 1989 Ratner *et al.*, 2006
4. Pneumococcal surface adhesin A	PsaA	Mediates adherence and colonisation Promotes resistance to oxidative stress by facilitating divalent metal uptake Binds host immune factor C3 and prevents apolactoferrin binding and killing by lactoferrin binding	Berry and Paton, 1996
5. IgA1 protease	-	Promotes pneumococcal adhesion and persistence at mucosal surfaces by cleaving IgA1, preventing IgA1-mediated pneumococcal clearance and exposing phosphorylcholine	Weiser *et al.*, 2003 Oggioni *et al.*, 2003 Batten *et al.*, 2003
6. Endonuclease A	EndA	Promotes pneumococcal invasion by degradation of NET DNA	Beiter *et al.*, 2006
7. Choline-binding protein E	CbpE	Promotes pneumococcal tissue dissemination	Attali *et al.*, 2008
8. Polyhistidine triad proteins D & E	PhtD/ BVH-11-2 PhtE/ BVH-3	Regulates complement deposition by recruitment of Factor H	Ogunniyi *et al.*, 2009
9. Pneumococcal serine-rich repeat protein	PsrP	Mediates epithelial attachment Promotes aggregate and biofilm formation	Sanchez *et al.*, 2010
10. Pneumococcal choline-binding protein A	PcpA	Promotes epithelial adherence	Khan *et al.*, 2012
11. Pneumococcal transformation pilus	ComGC	Facilitates DNA transformation by transient pore formation	Balaban *et al.*, 2014
12. Pneumococcal pilus 1 proteins	RrgA & B/ Rlr-regulated gene A	Promotes pilus-mediated epithelial adherence	Nelson *et al.*, 2007 Ahmed *et al.*, 2014
13. Pneumococcal pilus 2	PitB	Contributes to host cell adherence	Bagnoli *et al.*, 2008
14. Polyamine transporter D	PotD	Enables pneumococcal polyamine uptake for growth proliferation	Ware *et al.*, 2006
15. Neuraminidase A & B	Nan A & B/ Sialidase A & B	Mediates epithelial adherence Involved in biofilm formation	Brittan *et al.*, 2012 Parker *et al.*, 2009

Antigen	Abbreviation & alternative names	Role in virulence	References
16. Glycosyl hydrolase 25 relating to invasion protein	GHIP	Promotes nasopharyngeal colonisation and pneumococcal invasion and dissemination	Niu *et al*., 2013 Dong *et al*., 2014
17. Hyaluronate lyase	spnHL	Promotes pneumococcal dissemination by binding and cleaving hyaluronan residues of connective tissue extracellular matrix	Li *et al*., 2000
18. Pneumococcal protein endopeptidase O	PepO	Contributes to host cell invasion and immune evasion	Agarwal *et al*., 2013
19. Large zinc metalloproteinase	ZmpB	Role in inducing host inflammatory immune response	Blue *et al*., 2003
20. Cell wall-associated serine protease	PrtA	Cleaves apolactoferrin	Mirza *et al*., 2011
21. Pneumococcal adhesion and virulence A & B	Pav A & B	Modulates mediators of pneumococcal adherence, colonisation and invasion	Holmes *et al*., 2001; Pracht *et al*., 2005
22. Plasmin and fibronectin binding protein A	PfbA	Promotes adhesion and prevents pneumococcal phagocytosis	Yamaguchi *et al*., 2008
23. Pneumococcal iron acquisition/uptake A	PiaA/ PiuA	Promotes pneumococcal growth and survival	Brown, Gilliland and Holden, 2001
24. Autolysin B & C	LytB/ SP_46	Nasopharyngeal attachment and prevention of phagocytosis	Ramos-Sevillano *et al*., 2011
25. Protein for cell wall separation of GBS	PcsB	Role in cell division via Phospholipid phosphatidyl glycerol hydrolysis	Bajaj *et al*., 2015
26. Serine-threonine protein kinase	StkP	Involved in cell division	Fleurie *et al*., 2012
27. α-glycerophosphate oxidase	GlpO	Role in glycerol metabolism	Mahdi *et al* ., 2012
28. Maltose/maltodextrin-binding protein	MalX/ SP_2108/ GB144	Role in glycogen metabolism	Abbott *et al*., 2010
29. ABC transporter, substrate-binding protein	SPG_0148/ GB104	Role in carriage clearance	Moffitt *et al*., 2014
30. Hypothetical surface exposed protein	SPG_1912/ GB152	Role in carriage clearance	Moffitt *et a*., 2014

C3 – complement component 3; sIgA – secretory IgA; NET – neutrophil extracellular traps.

1.4.2.2.1 Pneumococcal surface protein A as a leading vaccine candidate

The Pneumococcal surface protein A (PspA) is a leading vaccine candidate due to its wide expression across all known pneumococcal strains (Crain *et al*., 1990), and ability to protect across serotypes (Tart *et al*., 1996). It is a surface-exposed protein which attaches the pneumococcal cell wall via its C-terminal choline binding domain (approx. 200 amino acids (aa)). PspA extends through the pneumococcal capsule with its flexible proline-rich linker region (approx. 100 aa), permitting surface exposure of the N-terminal helical module (300 – 400 aa) (Senkovich *et al*., 2007, Kolberg *et al*., 2003) (Figure 1.5a).

Murine models of pneumococcal infection have demonstrated PspA to play a role in protection from colonisation, otitis media, pulmonary infection and bacteraemia (Bogaert *et al*., 2004, Briles *et al*., 2000a). PspA functions to inhibit C3b deposition onto the pneumococcal surface, in turn blocking the progression of the alternative complement pathway in mediating pathogen clearance (Tu *et al*., 1999). PspA also binds lactoferrin: this competitively inhibits antimicrobial peptide apolactoferrin binding and killing *S. pneumoniae* (Ren *et al*., 2004).

PspA was first identified in monoclonal antibody screens and was found to be protective against fatal murine infection (McDaniel *et al*., 1984, McDaniel *et al*., 1986). Two decades on, PspA has exhibited safety and immunogenicity in phase I human vaccine trials (Briles *et al*., 2000a, Nabors *et al*., 2000). However, based upon computational sequence homology to human cardiac myosin, concerns over induction of autoimmunity has hampered its development. Further challenges include the sequence variability in PspA molecules: despite being conserved as a virulence factor, it is heterogeneous in sequence (Hollingshead *et al*., 2000). PspA has been classified into six clades and three families as per sequence homology: family 1 (clades 1 and 2), family 2 (clades 3 – 5) and family 3 (clade 6) (Hollingshead *et al*., 2000). 44% of human *S. pneumoniae* isolates are family 1, 55% family 2 and 1% family 3 (Briles *et al*., 2000b).

Darrieux and colleagues (2008) have demonstrated the ability of PspA fusion proteins constructed from a combination of the most abundant family 1 and 2 fragments, to induce homologous family cross-clade protection in a lethal murine challenge model. However, to

harness the full capabilities of PspA as a broadly protective vaccine candidate, novel vaccine strategies seek to elicit cross-family heterologous protection.

Multiple successive studies have highlighted the heterologous family protection abilities of immunisation strategies containing antigens from the most prevalent families 1 and 2. These include Briles and co-workers' (2000a) murine model demonstration of passive protection against lethal challenge with a heterologous PspA-expressing *S. pneumoniae* strain, following immunisation with human anti-PspA-Rx1 (clade 2, family 1)-containing serum. The following year, Kolberg and colleagues (2001) identified cross-family reactive monoclonal antibodies from *S. pneumoniae* strains from geographically distinct regions (Norway and The U.S.A).

Further still, Moreno *et al*. (2010) conducted a study which clearly illustrated the cross-reactive (antibody titre), cross-functional (mediation of C3 deposition) and cross-protective (intranasal challenge with heterologous PspA-expressing strains) capabilities of murine serum IgG specific to PspA clades 4 and 5 (family 2). This aligned with earlier immunogenicity studies which illustrated the cross-reactive properties of anti-PspA clade 4 and 5 antibodies raised in mice (Darrieux *et al*., 2007).

PspA from *S. pneumoniae* strain Rx1 (PspA/Rx1), is a well-characterised and studied protein by virtue of being the first PspA molecule to be sequenced and its protective domains identified (McDaniel *et al*., 1994, Talkington *et al*., 1991). Studies demonstrated the safety record of PspA/Rx1 in human volunteers, and post-immune serum contained antibodies which increased cross-clade functionality compared to pre-vaccination (Ochs *et al*., 2008, Nabors *et al*., 2000). Due to the foundation of information towards the utility of this strain, we employed PspA/Rx1 in protein vaccine candidate investigations.

We harnessed PspA fragments for immunological investigations due to their potential employment in downstream fusion protein approaches, and mitigation of potential PspA structural homology to human cardiac myosin due to reported low amino acid sequence homology (Ginsburg *et al*., 2012). Original work with these 100 aa PspA fragments (Figure 1.5b) by Vadesilho and co-workers (2014) investigated protection against pneumococcal pneumonia in a murine model on PspA immunisation. The protective epitopes located in the N-terminal alpha helical domain (Fragment 2: 79 – 178 aa) and the clade-defining region

(Fragment 4: 179 – 278 aa), elicited 100% and 67% protection respectively. No protection was afforded on immunisation with Fragment 7 (F7) of the proline-rich region (329 – 406 aa), whereas 100% protection was elicited by the parent protein PspA/Rx1.

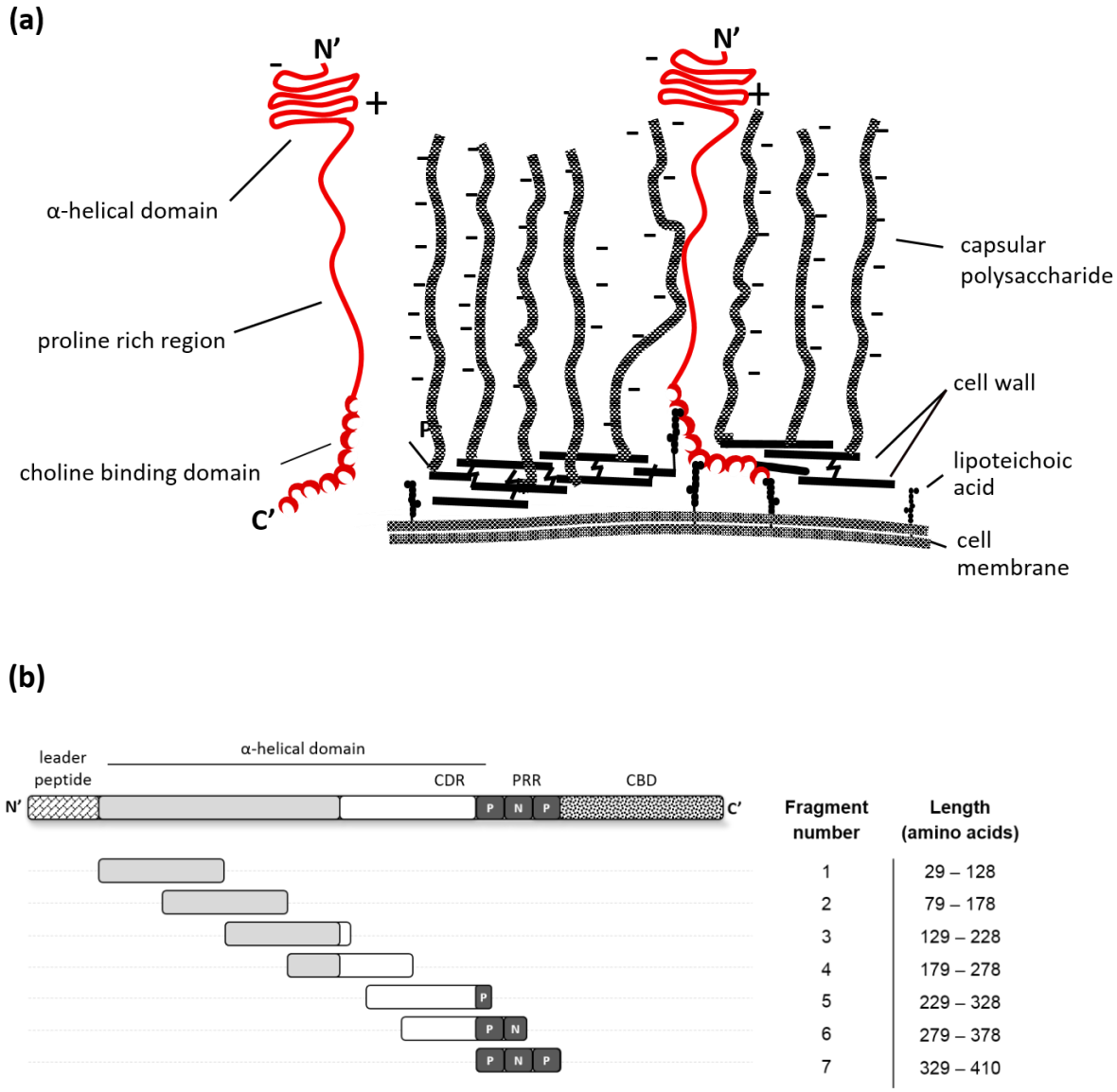


Figure 1.5 Pneumococcal surface protein A surface localisation illustration and PspA/Rx1 assay fragments schematic. (a) PspA attaches the *S. pneumoniae* membrane by binding choline-containing lipoteichoic acid (LTA) of the pneumococcal cell wall, protrudes through the capsule via its flexible proline-rich linker, enabling exposure of its functional alpha-helical module to the extracellular environment (b) Fragments 2, 4 and 7 were employed as vaccine candidates in *ex vivo* T-cell stimulation assays. CDR – clade-defining region, PRR – proline rich region (P – proline block, N – non-proline block), CBD – choline binding domain. Figure 1.5(a) courtesy of Professor D. Briles; Figure 1.5(b) adapted from Vadesilho *et al.*, 2014 [Adapted December 2016].

1.5 Natural immunity to Streptococcus pneumoniae

1.5.1 Innate defences

Innate immunity is the first line of defence against pneumococcal infection. Early and non-specific, it works in concert with the nasopharyngeal epithelium physical barrier to prevent pathogen invasion.

1.5.1.1 Antimicrobial peptides

Two abundant antimicrobial peptides involved in the respiratory epithelium innate defence against *S. pneumoniae* are lysozyme and lactoferrin (Travis *et al*., 2001).

Human lysozyme is present in secretions of both the upper and lower airway. It inhibits pneumococcal growth by muranidase activity, hydrolysing pneumococcal cell wall peptidoglycan. *S. pneumoniae* evades this innate attack by modification of its peptidoglycan structure through the action of enzymes PgdA, (an N-acetylglucosamine deacetylase) and Adr (an O-acetyl transferase) (Davis *et al*., 2008).

Lactoferrin is also present in nasal secretions: it sequesters iron necessary for bacterial growth, promotes bactericidal activity and immune modulation. Pneumococci evade killing by the toxic iron-depleted form, apolactoferrin, by competitively binding lactoferrin (Shaper *et al*., 2004).

1.5.1.2 Complement factors

Human complement deficiencies present a risk factor for invasive pneumococcal disease (IPD) (Picard and Casanova, 2003). C3 deficiencies result in recurrent cases of pneumococcal pneumonia (Buckley, 2004), demonstrating the importance of the complement system in preventing establishment of pneumococcal carriage and subsequent disease.

Complement proteins exist to clear host-invading organisms by opsonophagocytosis. The complement system is made up of three arms: the classical, alternative and lectin pathways. All converge on the activation of protein C3 to C3b which together with its processed product

iC3b, are deposited on the pneumococcal surface as opsonins promoting assembly of a membrane-attack complex and subsequent neutrophil-mediated phagocytosis (Walport, 2001a, Walport, 2001b).

Murine models of pneumococcal infection have illuminated roles of the complement pathways in pneumococcal diseases prevention. The classical pathway has been described as of importance in the activation of complement during the innate immune response to *S. pneumoniae* in mice with systemic infection through influence of C3 deposition proportions (Brown *et al*., 2002). The alternative pathway was implicated in the innate defence during a murine model of mucosal middle ear infection with a critical role of factor B, a protease required for alternative pathway convertase generation (Li *et al*., 2011). The lectin pathway has been described as playing a role driving survival against fatal murine infection via the essential role of the mannan-binding lectin-associated serine protease 2 (MASP-2) for lectin pathway convertase formation (Ali *et al*., 2012).

However, the pneumococcus has evolved numerous mechanisms to counter the three-pronged attack of the complement system. These include:

- Polysaccharide capsule expression - inhibition of C3 protein surface binding (Melin *et al*., 2010), prevention of C3b conversion to iC3b, and subsequent opsonisation via the alternative complement pathway (Hyams *et al*., 2010a)
- Surface protein expression - pneumococcal proteins that bind complement factors and serve as a decoy for the sequential workings of the complement system include;
    - Pneumococcal surface protein A, PspA - competes for phosphocholine binding with host defence factor C-reactive protein (CRP). In doing so, pneumococcal surface binding sites for CRP are occupied, and subsequent C3 deposition cannot occur (Mukerji *et al*., 2012)
    - Pneumococcal surface protein C, PspC - mediates immune evasion by binding Factor H which ordinarily attaches host residues glycosaminoglycan (GAG) and/or sialic acids, marking as self (Dave *et al*., 2001, Jarva *et al*., 2002). Contrarily to inhibition of C3 binding by CPS and PspA, PspC has the ability to bind complement protein C3, halting progression of complement

pathways (Cheng *et al*., 2000) and promoting epithelial cell adherence in the lung (Smith and Hostetter, 2000).

1.5.1.3 Toll-like receptors

On *S. pneumoniae* invasion, an innate mechanism of defence falls to the pattern recognition receptors (PRRs). A major class of PRRs are the Toll-like receptors (TLRs). Expressed on host cell surfaces, intracellularly, or secreted, PRRs recognise pathogen-associated molecular patterns (PAMPs) which are conserved microbial moieties that trigger inflammatory responses (Murphy *et al*., 2008).

Totalling ten in humans, three TLRs have been implicated in protection against pneumococcal colonisation and infection (Lee *et al*., 2007, Mogensen *et al*., 2006, Paterson and Orihuela, 2010):

- TLR2 - role in the protection against both pneumococcal carriage (van Rossum *et al*., 2005) and disease (Yoshimura *et al*., 1999). Major ligands have been identified as cell membrane lipoteichoic acid (Schroder *et al*., 2003), and lipoproteins (Tomlinson *et al*., 2014), which upregulate pro-inflammatory gene expression on binding (Krishnan *et al*., 2007).
- TLR4 - the major pneumococcal ligand is described as pneumolysin (Malley *et al*., 2003). Data on its role in protection from pneumococcal colonisation and disease is dichotomous. Malley and colleagues (2003) show the ability of potent pneumococcal virulence factor pneumolysin, to promote colonisation and invasive disease in TLR4 knock out mice, whereas McNeela *et al*. (2010) demonstrate a TLR4-independent mechanism of immune-induction against pneumococcal infection. Paterson and Orihuela (2010) suggest TLR4-directed protection as restricted to airway surfaces, and Branger *et al*. (2004) earlier demonstrate a role of TLR4 in defence against pneumococcal pneumonia.
- TLR9 - ligand described as unmethylated CpG repeats in DNA (Hemmi *et al*., 2000). Important in protection against pneumococcal lung infection (Albiger *et al*., 2007). Wang *et al*. (2008) demonstrated that morphine-induced impairment of TLR9

signalling dampens resident alveolar macrophage phagocytising abilities, resulting in a compromised immune response and greater susceptibility to pneumococcal infection.

1.5.1.4 Phagocytes

The increased susceptibility to pneumococcal infections in individuals with neutropenia (Rolston, 2001) points towards the importance of phagocytes in the immune defence against *S. pneumoniae*.

Circulating peripheral blood mononuclear cells (PBMCs) migrate to tissue both at steady state and in response to inflammation, where they develop into myeloid progenitor cells in the bone marrow. Myeloid progenitor cells are the precursor to multiple cell types, including phagocytic macrophages and neutrophils (Gordon and Taylor, 2005, Mosser and Edwards, 2008). Macrophages are tissue-specific: alveolar macrophages are the predominant cell in the human lung, and play a role in clearance of *S. pneumoniae* by opsonophagocytosis in the prevention of pneumonia (Jonsson *et al*., 1985, Dockrell *et al*., 2003), whilst splenic and liver macrophages are essential for systemic clearance and prevention of septicaemia (Gerlini *et al*., 2014).

Although diverse in specificity, macrophages maintain two core innate immune functions on encountering *S. pneumoniae*:

a) Generation of inflammatory responses to induce cellular recruitment and functions by cell surface TLR recognition of pneumococcal PAMPs, resulting in macrophage activation and inflammatory response induction (Lee *et al*., 2007)
b) Phagolysosomal pneumococci killing where differentiated macrophages fuse with *S. pneumoniae* containing lysosomes and induce killing by either:
    i. generation of microbicidal molecules e.g. reactive oxygen species (ROS), nitric oxide (NO) and reactive nitrogen species (RNS) (West *et al*., 2011)
    ii. macrophage apoptosis where phagolysosomes are permeabilised and the lysosomal protease cathepsin D activated (Bewley *et al*., 2011)

Neutrophils exert an enhanced phagocytic action compared to macrophages: this however, is *S. pneumoniae* opsonisation-dependent, with either complement, antibody or both (Hyams *et al*., 2010b, Yuste *et al*., 2008). Neutrophils are the most abundant leucocyte in peripheral blood: rapidly recruited to the site of infection where inflammatory mediators such as TNFα stimulate cells, which phagocytose *S. pneumoniae*. However, they exert detrimental effects to surrounding tissue in the process by production of ROS and proteinases by-products (Standish and Weiser, 2009). Furthermore, neutrophil degradation releases extracellular DNA, which together with histones and antimicrobial compounds, form neutrophil extracellular traps (NETs) (Brinkmann *et al*., 2004) to entrap and degrade *S. pneumoniae*. Pneumococci resist NET-mediated killing by virtue of their positive surface charge generating electrochemical repulsion against NET-entrapped antimicrobial peptides, and endonuclease EndA NET-degradation (Wartha *et al*., 2007, Beiter *et al*., 2006).

Innate immune mechanisms often trigger the adaptive. Mark J. Walport uses the example of the complement system to describe innate immune mechanisms as:

> *"… at the interface between innate and adaptive immunity"*
>
> (Walport, 2001b)

The inflammatory response that results from TLR-PAMP interaction serves too as an important link between innate and adaptive immunity, triggering inflammatory cytokine, chemokine and type I interferon secretion. This initiates the adaptive pathogen-specific and longer lasting B and T lymphocyte immune responses (Kumar *et al*., 2011).

1.5.2 Adaptive immunity

Over the time course of immunity, adaptive responses characteristically follow the initial innate. Adaptive immunity however, is highly pathogen specific and has memory; it is composed of both humoral and cellular responses.

1.5.2.1 Humoral immunity

The increased risk of pneumococcal infections amongst individuals with a human immunodeficiency where there are defects in immunoglobulin - such as the absence of Ig and depletion of circulating B-lymphocytes in X-linked agammaglobulinaemia (XLA) - provides insight into the important role of antibodies in the adaptive response against *S. pneumoniae* (Lederman and Winkelstein, 1985).

The humoral response results from the production of antibodies from B-lymphocytes. In humans, antibody production specific to pneumococcal proteins occurs naturally over a lifetime as a result of exposure to *S. pneumoniae* during nasal colonisation, and is demonstrated by increases in titres of the predominant serum antibody IgG, from childhood to adulthood (Rapola *et al*., 2000). Conversely, a decline in natural pneumococcal protein and capsular polysaccharide IgG and IgM antibodies is observed from young to older adults (Simell *et al*., 2008): this associates with increased disease susceptibility in the elderly.

The predominant human upper respiratory tract (URT) antibody is IgA subclass 1 (IgA1): IgA1 ordinarily binds the pneumococcus to prevent host epithelial adherence and promote phagocytosis (Weiser *et al*., 2003). *S. pneumoniae* however, has evolved mechanisms to circumvent this immune defence, the primary being cleavage of IgA by the IgA protease (Janoff *et al*., 2014). Roche and co-workers (2015) demonstrate in a passive transfer experiment, that human IgA1-immunised mice challenged with *S. pneumoniae* producing an IgA-1 protease did not agglutinate bacteria: this is where agglutination was identified as a pneumococcal clearance mechanism.

The role of antibodies in pneumococcal systemic and mucosal immunity after a previous colonisation episode has been demonstrated (Cohen *et al*., 2011). Circulating anti-pneumococcal IgG in mice previously colonised was the predominant antibody response and associated with reduced bacteraemia, whereas antibody knock-out mice sustained lethal bacteraemia. Bronchoalveolar lavage fluid (BALF) IgG and IgA were elevated in previously colonised mice: this associated with reduced bacteraemia in the lung.

1.5.2.2 T-lymphocyte cell-mediated responses

Antibodies play a recognised role in pneumococcal natural and vaccine-induced immunity. However, pneumococcal-specific T-lymphocyte-directed immunity, particularly $CD4^+$ T-helper cells, also play a role in pneumococcal carriage protection, mediation and clearance. Human T-cell deficiencies highlight the importance of the T-cell response to *S. pneumoniae*, where individuals are at an increased risk of respiratory tract infections (e.g. DiGeorge syndrome, a syndrome hallmarked by defective thymus development (Cole and Cant, 2010)), and pneumonia (e.g. Wiskott-Aldrich Syndrome (WASP) which results in a CD43 mutation therefore perturbed T-cell receptor signalling; and immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome where individuals have a FoxP3 mutation resulting in reduced T-regulatory function and autoimmunity (Ochs and Thrasher, 2006, Torgerson and Ochs, 2007).

$CD4^+$ T-cells are MHC class II-restricted lymphocytes, therefore preferentially recognise peptides 13 - 18 aa in length (Murphy *et al*., 2008). Antigen-specific, professional antigen presenting cells (APCs) e.g. dendritic cells, B-cells and macrophages, mediate peptide-MHC class II interaction.

Multiple murine model studies have elucidated mechanisms of T-cell immunity to pneumococcal infection. Protection from the pre-requisite to disease, pneumococcal colonisation, is described as antibody-independent (McCool and Weiser, 2004, Trzcinski *et al*., 2005, Malley *et al*., 2006), $CD4^+$ T-cell dependent (Malley *et al*., 2005, Basset *et al*., 2007) and antigen-specific (Trzcinski *et al*., 2008), whereas maintenance of the carriage state has been associated with T-regulatory cells (Neill *et al*., 2014, Zhang *et al*., 2011).

1.5.2.2.1 T-helper 1 cell immunity

T-helper 1 (Th1) immune responses are broadly pro-inflammatory and responsible for intracellular pathogen clearance. Characterised by the release of cytokine IFNγ, Th1 cells promote macrophage and neutrophil activation.

Humans with IFNγ deficiencies are more susceptible to *S. pneumoniae* infection. Kemp and colleagues (2002) demonstrated that patients with an *S. pneumoniae* infection as having a reduced systemic Th1 cytokine profile which improved on treatment.

Glennie *et al*. (2011) reported an increase in nasopharyngeal colonisation in individuals with symptomatic HIV (irrespective of antiretroviral therapy): these participants had a defect in pneumococcal-specific T-cell IFNγ production.

In vitro and murine experiments have further illuminated the role of Th1 responses to pneumococcal infection: where human monocytes and $CD4^+$ T-cells were co-cultured, live pneumococci demonstrated the ability to promote a Th1-biased, monocyte-driven immune response (Olliver *et al*., 2011). In a murine model of pneumococcal pneumonia, IFNγ was shown to play a protective role (Rubins and Pomeroy, 1997). Furthermore, Th1-directing adjuvants increase protection of conjugate immunisation in a murine systemic lethal infection model (Lefeber *et al*., 2003), and a neonatal lethal bacteraemia and lung pneumococcal infection model (Olafsdottir *et al*., 2012).

1.5.2.2.2 T-helper 2 cell immunity

T-helper 2 (Th2) immune responses are broadly associated with the humoral response against extracellular pathogens, and the counter to potential Th1 response-induced tissue damage. The primary cytokine hallmark of a Th2 environment is IL4 presence.

Dysregulation of Th2 responses are associated with allergy, including respiratory conditions such as asthma. This aligns with findings of reduced pneumococcal-antigen specific IgG titres in asthmatic children (Hales *et al*., 2012).

A distinct IL4 signature in the lungs of a mice with pneumococcal pneumonia highlighted a Th2 environment as a risk factor for infection (Kang *et al*., 2009). In an earlier study utilising a murine model of sinusitis, Th2 sensitisation increased severity of *S. pneumoniae* sinusitis (Yu *et al*., 2004).

1.5.2.2.3 T-helper 17 cell immunity

The T-helper 17 (Th17) immune response is pro-inflammatory and plays a role in the clearance of extracellular pathogens. The IL-17 family of proteins recruit and activate neutrophils, which subsequently clear infections. Although Th17 cells effectively hasten bacterial clearance of extracellular microbial pathogens (van de Veerdonk *et al*., 2009), they have been negatively implicated in chronic inflammation of autoimmune diseases (Oukka, 2008, Ouyang *et al*., 2008).

Th17 immunity has been described at the mucosal surface where the pro-inflammatory cytokine products of innate cell pathogen clearance upregulate Th17 responses, recruiting phagocytes to the site of infection and accelerating bacterial clearance (Figure 1.6).

Th17 responses play a role in the host's natural response to *S. pneumoniae*. Schmid *et al*. (2011) demonstrated Th17 immunity as the dominant immune arm in pneumococcal protein-specific immune response in human volunteers. On comparing two human populations differentially exposed to *S. pneumoniae*, a more robust pneumococcal protein-specific circulating Th17 response was found in the highly exposed population compared to the lower exposed population (Lundgren *et al*., 2012). Wright and colleagues (2013) detected human lower respiratory tract antigen-specific cellular responses to *S. pneumoniae* consistent with a Th17 phenotype.

Murine studies similarly illustrating the role of pneumococcal Th17 responses in protection from colonisation include Lu *et al*. (2008) and Cohen *et al*. (2011). Zhang and co-workers (2009) demonstrated the essential role of Th17 phenotype cells in clearance of both primary and secondary pneumococcal colonisation episodes, whereas Malley *et al*. (2006) demonstrated a role of IL-17A in protection against lethal pneumococcal pneumonia. More recently, Wang *et al*. (2017) demonstrate a robust Th17 response in the lungs of mice previously colonised and hence protected from pneumonia.

Antigens capable of inducing a Th17 response are therefore thought to be important in prevention of infection. Screening by Th17 induction has therefore been adapted as a selection method in identifying potential vaccine candidates (Moffitt *et al*., 2011).

1.5.2.2.4 T-regulatory cell immunity

T-regulatory (Treg) immunity plays an important role in the prevention of immune responses to self-antigen, non-harmful antigens and regulation of pathogen-induced immunopathology. Cytokine TGFβ is necessary for Treg induction (Huber *et al*., 2004) and therefore serves as a global marker of Treg activity together with IL-10.

Treg cells are broadly characterised by expression of transcription factor FOXP3, essential for phenotype development and cell function (Fontenot *et al*., 2003), and act to suppress the proliferation of $CD4^+$ and $CD8^+$ T-cells (Bacchetta *et al*., 2007). Antigen-specific Tregs (Tr1 or Th3 cells) are understood to play a role in prevention of disease. Treg induction has been demonstrated at mucosal surfaces with an inferred role in immune tolerance: this includes observations of *Bordetella pertussis*-specific responses in the respiratory tract of mice (McGuirk *et al*., 2002).

The immunosuppressive actions of Treg cells has been associated with the persistence of pathogens. Elevated numbers of Tregs in patients with multi-drug resistant *Mycobacterium tuberculosis* have been observed (Fan *et al*., 2016), with similar effects in the persistence of pneumococcal infection described with *S. pneumoniae*-induced Tregs. Neill *et al*. (2014) demonstrated elevated TGF-β1 and IL-10 levels in nasal washes of individuals positive for experimental human pneumococcal carriage compared to their carriage negative counterparts, and mice with low density, prolonged carriage episodes were shown to have a higher level of TGF-β1 compared to their high density comparators. In nasal associated lymphoid tissue (NALT), Zhang *et al*. (2011) report higher levels of Treg phenotype cells in carriage positive children compared to carriage negative individuals.

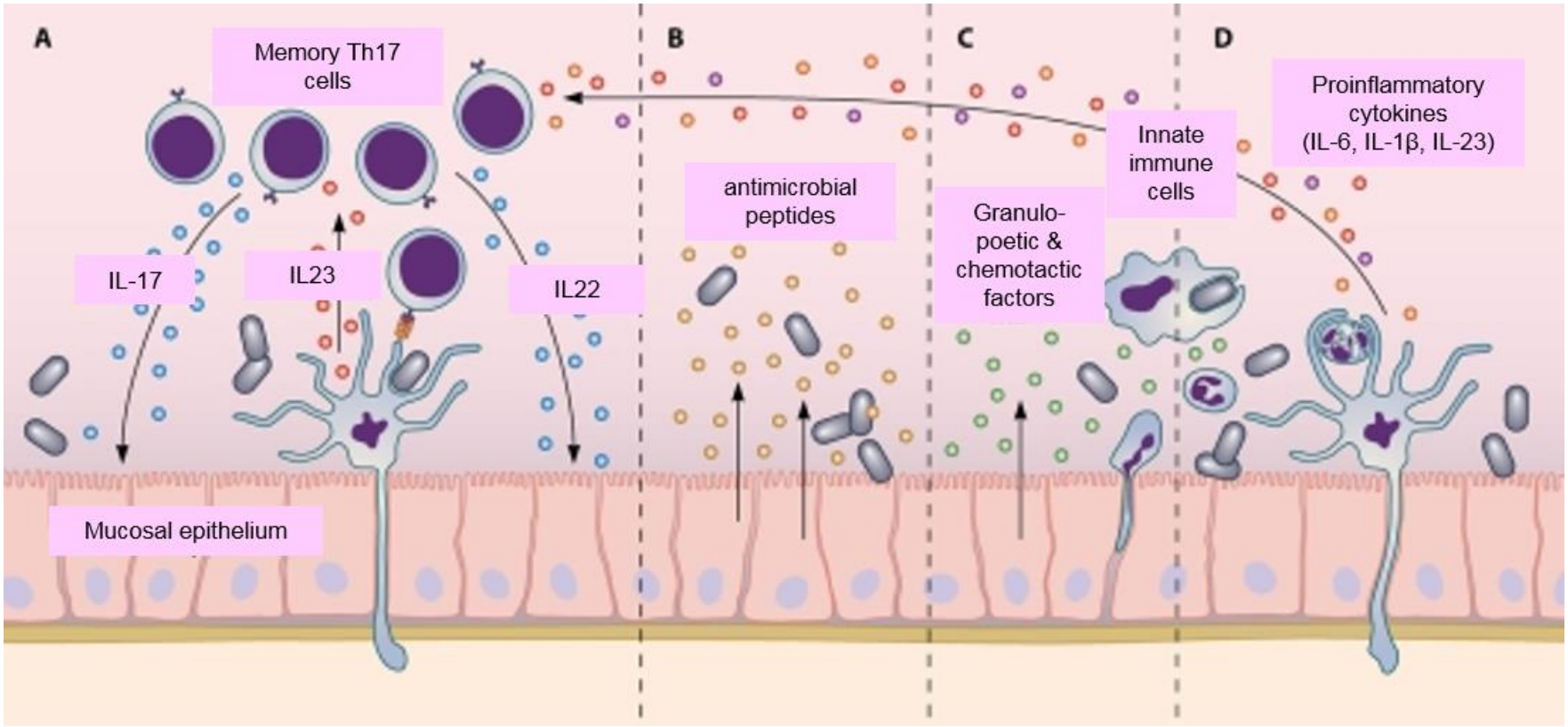


Figure 1.6 Mucosal infection and Th17 host defence at the mucosal epithelium (A) pathogens activate peripheral dendritic cells. IL-23 is produced which in turn upregulates resident memory Th17 cell IL-17 and IL22 production. (B) Epithelial cells produce antimicrobial peptides due to the synergistic effects of IL-17 and 22 inductive action. (C) IL-17 promotes epithelial cell expression of granulopoeitic and chemotactic factors. (D) Accumulation of innate immune cells at the mucosal epithelial surface promotes dendritic cell secretion of proinflammatory cytokines driving Th17 differentiation from naïve infiltrating T-cells. [Adapted from (Peck and Mellins, 2010); permission gained from American Society for Microbiology (ASM)].

Treg activity is observed reciprocally to Th17 immunity. Elevated numbers of Tregs in the adenoids of children positive for nasopharyngeal carriage and an inverse correlation to Th17 cells have been described (Jiang *et al*., 2015). Furthermore, *ex vivo* stimulation of NALT from carriage positive children has been show to yield a higher Treg: Th17 ratio than their carriage negative counterparts (Mubarak *et al*., 2016).

1.6 Vaccines and vaccine-induced immunity

A decreased ability to treat pneumococcal infections due to antibiotic resistance has been reported in the United Kingdom and worldwide (Guy *et al*., 2016, Appelbaum, 1992). The World Health Assembly resolution 67.25 on Antimicrobial Resistance calls on countries to develop national plans to fight antimicrobial resistance (Shallcross and Davies, 2014). The UK's five-year strategic plan response was launched in 2013 (Department of Health, 2013), results of which demonstrate a non-significant increase in penicillin non-susceptibility of *S. pneumoniae* from blood cultures (3.1% in 2010 to 4.4% in 2014) (Guy *et al*., 2016). The English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) (Public Health England, 2014), report a 23% reduction in pneumococcal bloodstream infections in a population with relatively high vaccine uptake (e.g. 93.6% for diphtheria-tetanus-pertussis, polio and *H. influenza* type b for children under 1 year, 2015-16 (NHS, 2016)). This highlights the importance of vaccination in disease prevention and subsequent last-line antibiotic preservation.

The first pneumococcal vaccine clinical trial was conducted amongst South African miners (Wright *et al*., 1914): highly susceptible to pneumococcal pneumonia, prophylactic treatment with whole pneumococci reduced pneumonia incidence and fatality. Three decades on, experiments by MacLeod and Hodges demonstrated that immunisation with *S. pneumoniae* capsular polysaccharide (CPS) prevented against pneumococcal pneumonia, highlighting the effectiveness of targeting CPS in vaccination. A clinical trial was conducted, and demonstrated the protective efficacy of a quadrivalent polysaccharide vaccine against pneumonia in a population of military recruits (MacLeod *et al*., 1945). However, with the co-incidental rise of

penicillin and antibiotic therapy, pneumococcal vaccine efforts were deserted (Klein *et al*., 2017).

Pneumococcal polysaccharide vaccination randomised clinical trials resumed in the 1970s (Austrian *et al*., 1976, Smit *et al*., 1977, Riley *et al*., 1977), and Merck licensed a 14-valent vaccine, expanded to a 23-valent in 1983 (Klein *et al*., 2017).

There are currently 94 known pneumococcal serotypes (Song *et al*., 2013). Antibodies generated against CPS protect against infection by mediating serotype-specific complement-dependent phagocytosis (Hostetter, 1986). Two vaccine types currently licensed are the purified polysaccharide and protein conjugated.

1.6.1 Purified polysaccharide

Pneumococcal polysaccharide vaccines are a preparation of purified CPS. The currently licensed contains twenty-three CPS serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F) and is so named the Pneumococcal Vaccine Polyvalent or PNEUMOVAX®23 (23vP). 23vP is indicated for the active immunisation for prevention of pneumococcal disease by the listed serotypes, is approved for use in children over 2 years of age, and adults over 50 years of age who are at risk of pneumococcal disease. It is supplied as a single 0.5 mL dose vial, or pre-filled syringe, to be administered intramuscularly or subcutaneously into the deltoid muscle or lateral mid-thigh (Pneumovax, 2017).

Pneumococcal CPS is a T-cell independent (TI) immunogen, and therefore elicits protection against infection with pneumococci of the 23 vaccine serotypes by generation of opsonising antibodies on stimulation of mature B-lymphocytes. Without T-cell help, memory is not induced and immune protection subsequently short-lived (Bridy-Pappas *et al*., 2005). The inability to mount a T-cell independent response limits vaccine efficacy, and is observed in the immunocompromised and extremes of age (Douglas *et al*., 1983).

23vP has limited effectiveness against pneumonia in the elderly (Huss *et al*., 2009). Booster doses resulted in hypo-responsiveness in adults, thought to be driven by an induced decrease

in memory and B1b-cell frequency, due to B1b antigens driving TI immunity (Clutterbuck *et al*., 2012). Studies on the clinical efficacy of PPV23 in HIV-infected adults have reported inconsistent findings, including one study where a higher risk of pneumonia was observed in vaccinees (Watera *et al*., 2004). Further still, the inability of the most vulnerable group to mount T-cell independent responses to immunogens (under 2s), means there is no demonstrable evidence of 23vP efficacy in very young children (Fedson, 1999).

1.6.2 Polysaccharide conjugates

Pneumococcal conjugate vaccines (PCV) are composed of CPS conjugated to a carrier protein. Proteins have included the tetanus protein, *Haemophilus influenzae* protein D, and the diphtheria toxoid (CRM-197). Conjugated CPS becomes a T-cell dependent immunogen, and therefore overcomes the downfalls of 23vP in that conjugate vaccines induce memory, protecting more effectively in vulnerable populations including healthy children under 2 years of age, and older at-risk children (Eskola *et al*., 2001, Darkes and Plosker, 2002).

The first pneumococcal conjugate vaccine to be licensed was the Polysaccharide Conjugate Vaccine 7, or Prevenar®7 (PCV7), protecting against pneumococcal disease from serotypes 4, 6B, 9V, 14, 18C, 19F and 23F (Black *et al*., 2000). Despite the efficacy of PCV7 in reducing the burden of vaccine-type disease, non-vaccine types continued to kill. Notably, where the disease burden was greatest in developing nations, protection against the two most common serotypes 1 and 5, was not included in the vaccination strategy. Therefore, serotypes 1, 3, 5, 6A, 7F and 19A were added to a formulation to create Polysaccharide Conjugate Vaccine 13, or Prevenar®13 (PCV13) (Jefferies *et al*., 2011).

In addition to post-PCV introduction reductions in worldwide burden of invasive pneumococcal disease (Rodgers and Klugman, 2016), PCV reduces rates of pneumococcal colonisation, reducing transmission and creating a herd immunity effect (Dagan and Fraser, 2000). Consequently, conjugate vaccines have an added benefit of reducing the total incidence of pneumococcal disease in unvaccinated community members.

A randomised placebo-controlled trial was conducted to evaluate the clinical benefit of PCV13 in the prevention of pneumococcal pneumonia in older adults (CAPiTA) and demonstrated a

halved vaccine efficacy of PCV13 against vaccine-type pneumococcal pneumonia compared to invasive disease (Bonten *et al*., 2015). This highlights the clinical need to understand biological mechanisms involved in protection from pneumococcal infection to improve vaccine efficacy.

Current vaccination strategies however, induce capsular polysaccharide-specific IgG which confers protection against pneumococcal disease in a serotype-specific manner. The phenomena of serotype replacement and capsular switching play a role in limiting vaccine effectiveness. Serotype replacement involves non-vaccine serotypes inhabiting the disease-causing niche vacated by vaccine-targeted serotypes (Gladstone *et al*., 2015), whereas with capsular switching the pneumococcus acquires a new capsular polysaccharide by horizontal gene transfer (Wyres *et al*., 2013). Both permit vaccine escape – where immunisation does not protect against non-vaccine serotypes (NVT).

Vaccine strategy has adapted to target emerging disease-causing serotypes, adding polysaccharides to vaccine formulation, evolving from PCV7 to the current PCV13. This however covers less than 15% of the potential 94 disease-causing serotypes. Together with the expense of conjugation, and potential of excessive polysaccharide antigens reducing the responsive B-cell population (Clutterbuck *et al*., 2012), the numbers of polysaccharides that can be added to formulation is limited.

Alternative strategies are therefore still needed, with a focus on mounting lasting serotype-independent protection. Understanding mechanisms by which the pneumococcus evades host immunity is important in the development of novel vaccination strategies.

1.7 Experimental human pneumococcal carriage

1.7.1 The model

The ability to investigate pneumococcal disease in the primary natural host, the human, is invaluable. Using the state of carriage as a surrogate for infection, novel vaccine candidates can be tested for their ability to prevent carriage, the pre-requisite to disease.

The Liverpool School of Tropical Medicine-based Experimental Human Pneumococcal Carriage model (EHPC) has been established such that 50% of healthy volunteers acquire carriage when intranasally inoculated with an average of 80,000 cfu/100 µL per naris of *S. pneumoniae* strain BHN418. Volunteers are carefully selected through a screening process, and closely monitored throughout the duration of a study by a safety committee of clinicians and scientists (Gritzfeld *et al*., 2013).

Individuals with immunity which prevents the carriage state can be viewed as the protected population, and those who become carriers, the susceptible population. This allows for probing of immunological factors influencing *S. pneumoniae* dynamics in the natural human host.

1.7.2 Biological and immunological utility

The EHPC model is a platform which has been used to investigate pneumococcal biology and the immunological factors regulating pneumococcal dynamics.

Humoral immunity affected by EHPC is described as increased pneumococcal-specific mucosal IgG on *S. pneumoniae* exposure (Wright *et al*., 2012), and augmented on carriage acquisition. We have reported two humoral correlates of colonisation protection: serotype 6B capsular polysaccharide IgG-secreting memory B-cells (Pennington *et al*., 2016), and capsular polysaccharide-specific mucosal IgG mediating *S. pneumoniae* agglutination (Mitsi *et al*., 2017). Elevated serum IgG with specificity to capsular polysaccharide and pneumococcal proteins conferred passive protection against murine lethal challenge with a heterologous pneumococcal strain (Ferreira *et al*., 2013). Cellular immune responses to carriage include that described by Wright and colleagues (2013) of increases in *S. pneumoniae*-specific $CD4^{+}IL\text{-}17^{+}$ T-cells in blood and the lung on carriage acquisition.

Biological factors associated with increased susceptibility to pneumococcal carriage acquisition were elucidated as diversity of nasopharyngeal microbiome, whereby increased diversity associated with increased rates of carriage (Cremers *et al*., 2014), and presence of an upper respiratory tract virus at the time of inoculation which increased rate of colonisation (Glennie *et al*., 2016). The biological state of pneumococcal carriage is protective against re-acquisition with a homologous *S. pneumoniae* (Ferreira *et al*., 2013).

Immunological mediators of *S. pneumoniae* carriage density and duration have been described. Increased nasal Factor H levels resulted in increased pneumococcal adherence via Pneumococcal surface protein C in an *in vitro* model of virus-pneumococcal co-infection (Glennie *et al*., 2016). The ability of nasopharyngeal cells to induce TGF-β1, together with T-regulatory cells, were shown by Neill and co-workers (2014) to play a role in the acquisition and maintenance of pneumococcal carriage.

The aforementioned studies contribute to our understanding of *S. pneumoniae carriage* as the pre-requisite for pneumococcal disease, and therefore inform strategies and targets of vaccination.

1.7.3 EHPC-PCV study: licensed vaccine efficacy against experimental carriage

The EHPC platform has been used to assess vaccine efficacy as measured by pneumococcal colonisation. Vaccine clinical trials are often expensive and lengthy due to the number of individuals required to investigate population-level efficacy against disease. To validate the EHPC platform for use in vaccine efficacy, Collins and colleagues (2016) determined the effect of the licensed PCV-13 on colonisation with vaccine serotype 6B in a double blind randomised controlled trial.

During this study of 100 volunteers, the vaccinated group had a significantly reduced colonisation rate (risk ratio = 0.22, CI = 0.09 to 0.52, $p < 0.01$), and density and duration of colonisation (geometric mean vaccinated: 259, geometric mean placebo: 11183, $p = 0.02$) compared to the placebo vaccinated-group. Therefore, the EHPC model was demonstrated as an effective method of assessing the protective efficacy of novel vaccine on pneumococcal colonisation.

Notably, the EHPC model has proven safe, efficient and cost-effective in investigating effects of the licensed vaccine PCV13 on nasopharyngeal colonisation (Collins *et al*., 2016): it therefore offers a platform to test the effect of novel vaccines on colonisation and associated immunological parameters.

1.7.4 GEN-004: new vaccine efficacy against experimental carriage

The EHPC platform has been used to investigate the safety, immunogenicity and efficacy of Genocea biosciences' novel vaccine candidate GEN-004. Containing three T-cell inducing universal pneumococcal proteins: maltose/maltodextrin-binding protein (SP2108), ABC transporter substrate-binding protein (SP0148), and hypothetical surface exposed protein (SP1912), GEN-004 was protective against pneumococcal carriage in murine models (Moffitt *et al*., 2014).

During the Phase II study using the EHPC platform, GEN-004 was reported as safe and immunogenic as determined by antibody titres to vaccine antigens: 100% of volunteers responded to antigen SP2018, 97.7% to SP0148, and 83.7% to SP1912. GEN-004 was also demonstrated as capable of modest reductions in volunteers with experimental carriage and volunteer carriage density as determined by classical microbiology (day 2 colonisation rates: 52% vaccinated, 39% placebo) and qPCR. However, no result was statistically significant between placebo and vaccine immunised groups (Skoberne *et al*., 2016).

1.8 New pneumococcal vaccines

1.8.1 Whole cell vaccine

Whole cell vaccines such as the unencapsulated *S. pneumoniae* strain developed by PATH (formerly Program for Appropriate Technology in Health), expose surface proteins common to multiple serotypes as immunogens. Pre-clinical murine trials of PATH's whole cell *S. pneumoniae* vaccine (wSP) have shown protection against colonisation and invasive disease by multiple pneumococcal strains (Malley *et al*., 2001, Malley *et al*., 2004).

To date, two Phase I trials of PATH wSP have been completed in the U.S.A and Kenya to identify the optimal vaccine dose in adults and toddlers, in addition to a Phase II efficacy study on nasopharyngeal carriage in the paediatric Kenyan population. A third trial in the Kenyan cohort is underway, and is a Phase II to assess PATH wSP safety and immunogenicity as measured by serology (U.S. National Institutes of Health, 2014).

1.8.2 Recombinant bacteria

Recombinant bacteria expressing pneumococcal antigens protect mice against infection to varying extents. Their attraction as a vaccine strategy lies in their low-cost vaccine production, needle-free delivery, and induction of strong mucosal immunity (Curtiss *et al*., 2010).

Xin *et al*. (2009) utilise a recombinant avirulent *Salmonella enterica* serovar Typhimurium vaccine system (RASV) for expression and secretion of PspA fusion proteins: immunised mice were protected against infection with *S. pneumoniae* clinical strains expressing a heterologous PspA. An extension of the RASV system is described and termed regulated delayed antigen synthesis (RDAS) (Wang *et al*., 2010). Protection against challenge with an *S. pneumoniae* strain expressing a heterologous PspA is enhanced on regulation of PspA expression, reflected in reduced IgG titres in the RASV strain constitutively expressing PspA.

Surface expression of PspA by heterologous bacteria has also proven effective in reducing *S. pneumoniae* infection. Kothari *et al*. (2015) model a system of conjugation of PspA to Salmonella Typhi Vi capsular polysaccharide, protective against lethal challenge in murine models. Furthermore, Kuipers and co-workers (2015), demonstrated protection against *S. pneumoniae* colonisation upon immunisation with PspA-expressing salmonella outer membrane vesicles.

An early example of a similar inducible expression system was developed by Oliveira and colleagues (2003), where *Lactobacillus casei* is the vector, and PspA the test antigen. PspC has also been expressed using *L. casei*, and exposed on the bacterial surface on nasal immunisation, resulting in induction of high mucosal anti-PspC IgA titres in mice protected from colonisation (Hernani *et al*., 2011). Live lactic acid bacteria are an attractive vector as they can be used to deliver protective antigens mucosally and have been shown to elicit mucosal and systemic antigen-specific immunity (Vintini and Medina, 2011).

Optimisation of recombinant protein vaccine systems is important in the arena of new protein vaccines vaccine discovery: realising methods of antigen delivery for maximum protective effect is vital in the identification of novel, immunogenic and protective pneumococcal protein vaccine candidates.

1.8.3 Protein vaccine candidates

Vaccine-driven changes in the pneumococcal population are challenges to lasting protection against pneumococcal disease. The primary hurdle is the phenomenon of serotype replacement, but also includes capsule switching and increase in serotype diversity. The latter is eloquently compared to the Red Queen hypothesis (reciprocal co-evolution of competing species, human beings and bacteria) (Stockmann *et al*., 2016). In all cases, non-vaccine capsular types pose an increased risk to causing infection and disease.

The hurdle of serotype-specific, short-lived vaccination strategies together with the high costs of manufacture, has made the development of new cost-effective, broad coverage pneumococcal vaccines a global health priority (Miyaji *et al*., 2013). Pneumococcal protein vaccine approaches are designed to address this need, with the most promising candidates alongside their roles in pneumococcal virulence tabulated in Table 1.1.

In addition to vaccination, *S. pneumoniae* protein constructs are under investigation for immunotherapy strategies. One such example is P4, a 28 aa construct of the Pneumococcal surface adhesion A protein (PsaA) (Rajam *et al*., 2008). Bangert and colleagues (2013) demonstrated efficacy towards promotion of *ex vivo S. pneumoniae* phagocytic killing on treatment of human alveolar macrophages. Furthermore, clinical samples obtained from adults with severe sepsis infection demonstrated the effect of P4 peptide on enhancing neutrophil phagocytosis of *S. pneumoniae* and hence, its potential use as an augmented immunotherapy (Morton *et al*., 2016).

Protein vaccines composed of single and multiple surface antigen formulations of antigens widely expressed across pneumococcal strains are known to induce adaptive immunity above capsular polysaccharide (Moreno *et al*., 2010, Cohen *et al*., 2013). Together with the potential to overcome barriers of serotype replacement and lack of mucosal efficacy, we chose to investigate protein-specific immune responses to *S. pneumoniae* in healthy adult volunteers using the Experimental Human Pneumococcal Carriage model.

1.9 Study aims

Our overall aims and specific objectives were to:

1. **Further our current understanding of humoral PspA-specific responses to *S. pneumoniae***

- Investigate whether protection against re-acquisition of carriage associates with *S. pneumoniae* expressed PspA family
- Assess if pneumococcal exposure alters heterologous PspA family IgG titre
- Assess whether pneumococcal exposure modulates IgG binding to *S. pneumoniae* expressing heterologous PspA families

2. **Investigate blood PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae***

- Determine whether antigen-specific $CD4^+$ T-cell responses to *S. pneumoniae* are detectable in blood
- Assess whether inoculation alters antigen-specific $CD4^+$ T-cell responses to *S. pneumoniae*
- Compare antigen-specific $CD4^+$ T-cell responses to *S. pneumoniae* post-inoculation across carriage status
- Examine whether antigen-specific $CD4^+$ T-cell responses to *S. pneumoniae* associate with colonisation intensity

3. **Investigate BAL PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae***

- Compare non-PspA-specific pulmonary $CD4^+$ T-cell cytokine levels across carriage status
- Determine whether PspA-specific immune response to *S. pneumoniae* is detectable in pulmonary $CD4^+$ T-cells
- Explore if carriage augments PspA-specific responses to *S. pneumoniae* in pulmonary $CD4^+$ T-cells

- Assess whether PspA-specific responses to *S. pneumoniae* in pulmonary $CD4^+$ T-cells correlate with carriage intensity
- Investigate whether PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* are of comparable magnitude in the lung and blood

4. Investigate systemic *S. pneumoniae* epitope-induced lymphoproliferation

- Identify the thirty most studied, immunogenic pneumococcal protein vaccine candidates by literature search
- Determine conserved peptide sequences of functional importance (fragility islands) within selected vaccine candidates
- Optimise lymphoproliferation assay conditions
- Assess lymphoproliferation activity of selected epitopes

CHAPTER 2

General methods

2 General methods

Investigating human protein-specific immune responses to *S. pneumoniae* required a number of methods. Specifically: volunteer recruitment and ethical approval, The Experimental Human Pneumococcal Carriage model, obtaining and processing of clinical samples, and laboratory procedures. Methods and materials common to more than one study chapter are detailed below, with an overview recapitulated in the relevant thesis chapter. Study-specific methods and materials are detailed in the relevant thesis chapter.

2.1 Volunteer recruitment and ethical approval

All four studies in this thesis (chapters 3 – 6) required human volunteer participation. Therefore, participants were recruited subject to ethical approval.

2.1.1 Experimental Human Pneumococcal Carriage studies

Healthy adult volunteers were enrolled with written informed consent to an Experimental Human Pneumococcal Carriage study (chapters 3 to 5) with inclusion criteria:

- 18 - 60 years of age;
- proficiency in the English language;
- communicable by electronic means (telephone and text message).

Volunteers were unable to participate if they fell under any exclusion criteria:

- close contact with at-risk individuals (young children, immunosuppressed adults, elderly adults, individuals with chronic ill health);
- current smoker or a significant smoking history (> 10 pack years);
- sufferer of asthma or other respiratory disease;
- pregnant;
- allergic to penicillin;
- involved in another clinical trial (unless observational or in a non-interventional phase);

- unable to give full informed consent.

Ethical approval was obtained from the National Health Service Research Ethics Committees (REC 12/NW/0873 (EHPC-PCV clinical trial) and 11/NW/0592 (Dose-ranging/Re-challenge study)), and studies sponsored by the Royal Liverpool and Broadgreen University Hospitals Trust. Clinical trial approval (EHPC-PCV) was granted by the Medicines and Healthcare products Regulatory Agency (MHRA) (EudraCT 2012-005141-20). Volunteer appointments took place in the Clinical Research Unit at the Royal Liverpool University Hospital.

2.1.2 Epitope-induced lymphoproliferation study

Healthy adult volunteers were recruited from the Liverpool School of Tropical Medicine blood donor register under the Research Tissue Bank ethics approval granted by The North West 3 Research Ethics Committee, Liverpool East (REC 11/H1002/9) and Liverpool School of Tropical Medicine Human Tissue Act Research Licence number 12548. Volunteer appointments took place in the Department of Clinical Sciences at the Liverpool School of Tropical Medicine.

2.2 The Experimental Human Pneumococcal Carriage model

The Experimental Human Pneumococcal Carriage (EHPC) model utilised clinical isolate *S. pneumoniae* serotype 6B strain BHN418 as the inoculation strain (GenBank accession number ASHP00000000.1) (a gift of P. Hermans of Radboud University, Nijmegen, S. Normark and B. Henriques-Normark of the Karolinska Institute, Stockholm). An animal product-free medium, Vegitone Infusion broth (Fluka, Buchs, Switzerland), was used to grow the inoculum stock, and its serotype, penicillin sensitivity, and purity independently confirmed by the Public Health England reference laboratory. Each inoculum was prepared on the day of inoculation and dose quantified to an average 80,000 colony-forming units (cfu)/ 100 µL per naris, as quantified by Miles and Misra (Miles *et al*., 1938).

Nasal carriage was determined before and after inoculation by nasal wash sampling (Gritzfeld *et al*., 2013). Volunteers seated in a semi-recumbent position had 5 mL sterile saline inserted

into their anterior nasal space, which was immediately expelled by rapid exhalation and collected into a foil bowl. Saline insertion-exhalation cycles were repeated until 20 mL saline had been washed through the nose.

Pooled samples were pelleted and plated onto chocolate blood agar (CBA) for pneumococcal carriage determination. Gentamicin (4 µg/mL) CBA was used to negatively select against commensal microbial flora. Suspected *S. pneumoniae* colonies were subcultured for purity, Gram stain, optochin sensitivity and bile solubility. *S. pneumoniae* serotype was determined by latex agglutination (Statens Serum Institute, Denmark), and density in cfu/mL of returned nasal wash (Gritzfeld *et al*., 2013).

The Dose-ranging/Re-challenge study pneumococcal preparation, inoculation and subsequent detection was carried out as described above, with the modification of five extra doses. Six groups of 10 volunteers were inoculated with increasing target doses of *S. pneumoniae* strain BHN418: 1, 2, 4 and 8 $x10^4$, 1.6 and 3.2 $x10^5$ cfu/100 µL per naris.

2.3 Clinical samples: obtaining and processing

2.3.1 EHPC-PCV clinical trial

Nasal wash samples were obtained for determination of natural pneumococcal colonisation at 5 days before inoculation. Experimental carriage was determined by classical microbiology at days 2, 7 and 14 following inoculation with a single intranasal target dose of 80,000 cfu/100 µL per naris *S. pneumoniae* serotype 6B (strain BHN418).

Blood samples were obtained 5 days before and 21 days after *S. pneumoniae* inoculation. Peripheral blood mononuclear cells (PBMCs) were isolated by density-mediated centrifugation using Ficoll-Paque PLUS (GEHealthcare, Uppsala (Sweden)) according to the manufacturer's instructions. PBMCs were washed twice in PBS, re-suspended in complete media [RPMI $^+$ 20 mM L-glutamine supplemented with antibiotics (penicillin 40 U/mL, streptomycin 40 µg/mL, neomycin 80 µg/mL) and 10% Foetal Calf Serum (FCS) (Invitrogen)], and seeded into a 24-well plate in a volume of 1 mL and concentration of 1 x 10^6 PBMCs/mL.

Research bronchoscopy and Bronchoalveolar lavage (BAL) was offered to participants between weeks 9 – 21 on completion of the study. This followed administration of a 3-day course of Amoxicillin 500 mg if they were carriage positive (Figure 2.1a).

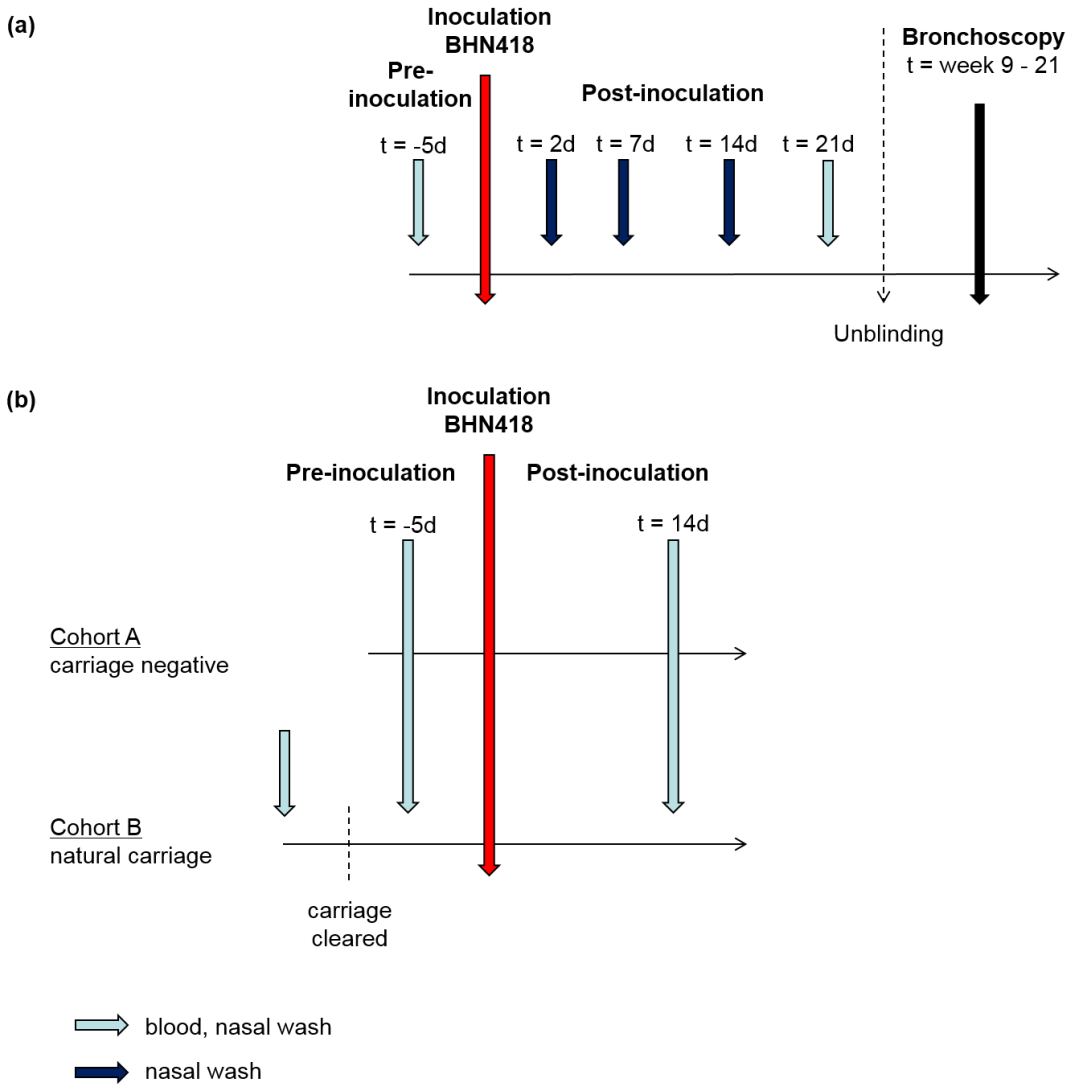


Figure 2.1 Timeline for sample collection relative to inoculation with *S. pneumoniae* strain BHN418 (serotype 6B expressing PspA1) (a) EHPC-PVC clinical trial: blood samples were taken 5 days pre- (t = -5d) and 21 days post-inoculation (t = 21d). Nasal washes taken at intermediary days (d) 2, 7 and 14 for monitoring of pneumococcal carriage (carriage negative, c-, = 32; carriage positive, c+, = 8). Bronchoscopy offered (week 9 – 21) after unblinding for carriage status (c- = 6, c+ = 8). (b) Dose-ranging/Re-challenge study: serum samples obtained from blood 5 days pre- (t = -5d) and 14 days post-inoculation (t = 14d). Cohort A (c- = 16, c+ = 12); Cohort B (natural carriers at baseline) pre-inoculation samples obtained after clearance of natural carriage episode (post-inoculation c- = 4, c+ = 4). Natural carriage isolates molecularly typed to identify PspA clade and family.

Whole BAL samples were filtered through muslin to remove mucus plugs and 5 mL of unprocessed BAL stored at −80°C (Figure 2.2). Whole sample was pelleted at 470 x *g* for 10 minutes; supernatant was stored at −80°C for subsequent humoral analysis, and the cellular fraction taken forward for *ex vivo* stimulation. Cells were re-suspended in complete media and differentially counted to note macrophage and lymphocyte yield, plated into standard 6-well tissue culture plates at a concentration of 5 x 10^5 macrophages/mL, and incubated at 37°C, 5% CO2 for 3 hours to promote macrophage adhesion. Non-adherent cells were collected, washed in complete media, and plated at a count of 200 – 500,000 cells/mL in a 96-well plate.

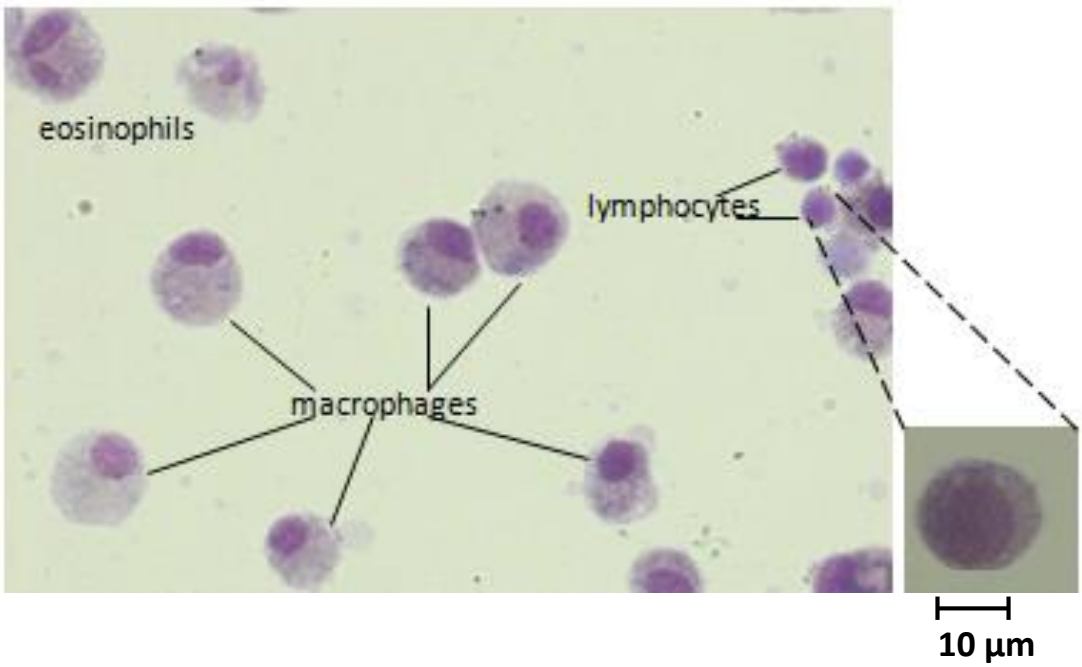


Figure 2.2 Whole BAL cytospin differential stain to demonstrate immune cells found in the lung. Alveolar macrophages represent one of the most abundant immune cells in the lung which were extracted by adhesion, and lymphocytes (inset) a population of non-adherent cells. Horizontal bar represents 10 µm in length.

2.3.2 Dose-ranging/Re-challenge study

Nasal wash samples were obtained for determination of natural pneumococcal colonisation 5 days before inoculation. Experimental carriage was determined by classical microbiology, 14 days after inoculation with *S. pneumoniae* strain BHN418.

Serum samples were obtained from blood 5 days before and 14 days after *S. pneumoniae* inoculation. Healthy adult volunteers were stratified into two groups and inoculated with a target dose of 40,000 cfu/100 µL per naris: Cohort A, negative for carriage on presentation, and Cohort B, who were naturally colonised with *S. pneumoniae* at presentation (re-inoculated after clearance of their natural carriage episode) (Figure 2.1b).

2.3.3 Epitope-induced lymphoproliferation study

Blood samples were collected at a single time-point for PBMC isolation and subsequent lymphoproliferation experiments. Peripheral blood mononuclear cells (PBMCs) were isolated by density-mediated centrifugation using Ficoll-Paque PLUS (GEHealthcare, Uppsala (Sweden)) according to the manufacturer's instructions. PBMCs were washed twice in PBS, re-suspended in complete media [RPMI + 20 mM L-glutamine supplemented with antibiotics (penicillin 40 U/mL, streptomycin 40 µg/mL, neomycin 80 µg/mL) and 10% Foetal Calf Serum (FCS) (Invitrogen)], and seeded into a 24-well plate in a volume of 1 mL and concentration of 1 x 10^6 PBMCs/mL.

2.4 Laboratory procedures

To investigate protein-specific immune responses to *S. pneumoniae*, we synthesised pneumococcal proteins and peptides. A leading vaccine candidate, Pneumococcal surface protein A (PspA), and derivative fragments were employed as stimulants in $CD4^+$ T-cell assays of chapters 4 and 5.

2.4.1 Recombinant protein expression and purification

The PspA of *S. pneumoniae* strain Rx1 (PspA/Rx1) (GenBank accession number M74122.1) was selected as it is well characterised having been the first PspA molecule to be described (Kolberg *et al*., 2003, McDaniel *et al*., 1994). The 100 aa fragments, with a 50 aa overlap covering the mature N-terminal region of the proline-rich region, were constructed and purified as previously described (Vadesilho *et al*., 2014).

2.4.1.1 Bacterial strains and plasmids

Escherichia coli BL21/DE3 (Sigma-Aldrich®, St Louis (USA)) was used as the host strain for all protein expression experiments. His-tagged PspA/Rx1 fragments originating from *S. pneumoniae* strain Rx1 were engineered into ampicillin-selectable and IPTG-inducible expression vector pAE (Ramos *et al*., 2004) to produce plasmids pAE-*pspARx1* (1,011 bp), pAE-*frag 2* (300 bp), pAE-*frag 4* (300 bp) and pAE-*frag 7* (243 bp) (Vadesilho *et al*., 2014).

Recombinant *E. coli* was cultured in Luria Bertani (LB) broth (Sigma-Aldrich®, St Louis (USA)) supplemented with 1 µg/mL ampicillin. Incubation at 37°C at an agitation of 180 rotations per minute (rpm) for 18 – 24 hours constituted an overnight (O/N) culture.

2.4.1.2 Transformation of heat competent *E. coli*

Competent BL21(DE3)-T1R cells (Sigma-Aldrich®, St Louis (USA)) of 50 µL were thawed on ice for 15 minutes, 5 µL of PspA/Rx1 DNA added and left on ice for 30 minutes. Cells were given a thermal shock at 42°C for 2 minutes and recovered in 500 µL of Super Optimal broth with Catabolite repression (SOC) [0.5% yeast extract (Sigma-Aldrich®); 2% Tryptone (Sigma-Aldrich®); 10 mM NaCl (Sigma-Aldrich®); 2.5 mM KCl (BDH); 10 mM MgCl2 (BDH); $MgSO_4$ (Sigma-Aldrich®); 20 mM glucose (BDH)] at 37°C and 180 rpm for 1 hour. Dilutions of 50, 100 and 300 µL were plated onto ampicillin-selective LB agar plates, and incubated at 37°C O/N to confirm successful transformation.

2.4.1.3 Induction of His-tagged protein

O/N cultures of each bacterial clone were subcultured in a 1:10 dilution of LB broth (Sigma-Aldrich) supplemented with 1 µg/mL ampicillin, and grown to an optical density (OD) of 0.6 – 0.7 at wavelength 600 nm (OD_{600}). Protein expression was induced by addition of 1M IPTG and incubated at 37°C for 3 hours. Cells were isolated by centrifugation at 1,300 x *g* for 20 minutes, supernatants discarded and pellets stored at -20°C.

2.4.1.4 Cell lysis

Bacterial cell pellets containing expressed protein were thawed on ice and underwent cell lysis by one of two methods: mechanical disruption (proteins used in Chapter 4) or enzymatic lysis (proteins used in Chapter 5).

2.4.1.4.1 Mechanical disruption

Cells were re-suspended in equilibration buffer [50 mM Tris pH 6 - 8 (FischerScintific), 150 mM NaCl (Sigma-Aldrich®), 5 mM imidazole (Sigma-Aldrich®)]. Cells were then disrupted at a pressure of 130 bars (1885.5 psi) using the PandaPLUS 2000 system (GEA Niro Soavi, Parma, Italy).

2.4.1.4.2 Enzymatic lysis

Lysozyme and Benzonase® Nuclease supplemented lysis buffer (Qiagen) was used to lyse cells by incubation at 4°C for 30 minutes.

2.4.1.5 Protein purification and dialysis

Cell lysis cellular debris was removed by centrifugation at 14,000 x *g* and the resultant soluble expressed fraction purified using Ni-NTA Resin (Qiagen) according to a previously described QIA*express*® Ni-NTA Fast Start Handbook protocol (June 2006) using in-house wash buffers of varying imidazole concentration [stock: 15 mL 1M Tris pH 8.0 (FisherScientific), 9 mL 5M NaCl (Sigma-Aldrich®), 1M imidazole (Sigma-Aldrich®) (1.5 mL for 5 mM wash buffer; 6 mL for 20 mM; 12 mL for 40 mM; 18 mL for 60 mM; 75 mL for elution buffer) completed to 300 mL with MilliQ H2O].

Purified proteins were dialysed using 7K MWCO SnakeSkin™ dialysis tubing (ThermoScientific, Rochford, (USA)) to remove imidazole and other contaminant salts according to a previously described manufacturer's protocol (2013), and quantified by densitometry using SDS gel electrophoresis reads and GelAnalyzer2010® software (Lazar, 2017).

2.4.2 *Ex vivo* stimulation

Purified proteins were used as stimulants in investigation of PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* in blood (chapter 4) and BAL (chapter 5).

2.4.2.1 Blood

Isolated PBMCs were left unstimulated (for background subtraction of non-specific cytokine secretion), or separately stimulated with 4 µg/mL PspA antigens (PspA/Rx1, Fragments 2, 4 and 7), or 0.5 µg/mL positive assay control staphylococcus enterotoxin B (SEB). PBMCs were incubated at 37°C, 5% CO_2 for 120 hours and in the final 16 hours of stimulation, 1 µL of a 3 mM monensin stock (BD Biosciences, San Diego (USA)) and 1 µL of a 1mg/mL brefeldin stock (BD Biosciences) were added to each well, preventing release of induced cytokines from the endoplasmic reticulum and Golgi apparatus of lymphocytes.

2.4.2.2 BAL

Non-adherent cells were left unstimulated (for background subtraction of non-specific cytokine secretion), or separately stimulated for 16 hours with 8 µg/mL PspA antigens PspA/Rx1, Fragments 2, 4 and 7. After incubation for 2 hours at 37°C, 5% CO_2, 1 µL of a 3mM monensin stock (BD Biosciences, San Diego (USA)) and 1 µL of a 1mg/mL brefeldin stock (BD Biosciences) were added to each well, preventing release of induced cytokines from the endoplasmic reticulum and Golgi apparatus of lymphocytes.

2.4.3 Flow cytometry

Intracellular cytokine staining (ICS) was employed to monitor PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* (Chapters 4 and 5).

Cells were harvested by centrifugation and stained for surface phenotyping and intracellular cytokine production. 300,000 lymphocyte cell events (blood) and 100,000 (BAL) were acquired using a BD LSRII Flow Cytometer (Becton Dickinson). Cells were viewed using FacsDiva

software version 6.1 (BD Biosciences) in the LSTM Flow Cytometry Facility. Viable $CD3^+CD4^+CD69^+$ T-cells (hereafter described as activated $CD4^+$ T-cells) and IL-17A, IL-22, TNFα, IFNγ and TGFβ secretors were identified and profiled. The gating strategy is described in Figure 2.3.

2.4.4 Statistical analysis

Cytokine-secreting $CD4^+$ T-cells measured in ICS assays were quantitively analysed (chapters 4 and 5). Raw percentage cytokine-secreting $CD4^+$ T-cell data underwent initial analysis by One-way ANOVA with Dunnett's post-hoc testing. Subsequent exploratory analysis employed normality testing on percentage cytokine-secreting $CD4^+$ T-cell data, by Sharipo-Wilk analysis (Kolomogorov-Smirnov for smaller datasets). Cohort data following a Gaussian distribution underwent parametric analysis, namely unpaired t and Pearson's correlation tests. Graphical representation and statistical analyses were performed using GraphPad Prism v5.0 (California, USA). Differences were considered significant at $p < 0.05$ (two-tailed).

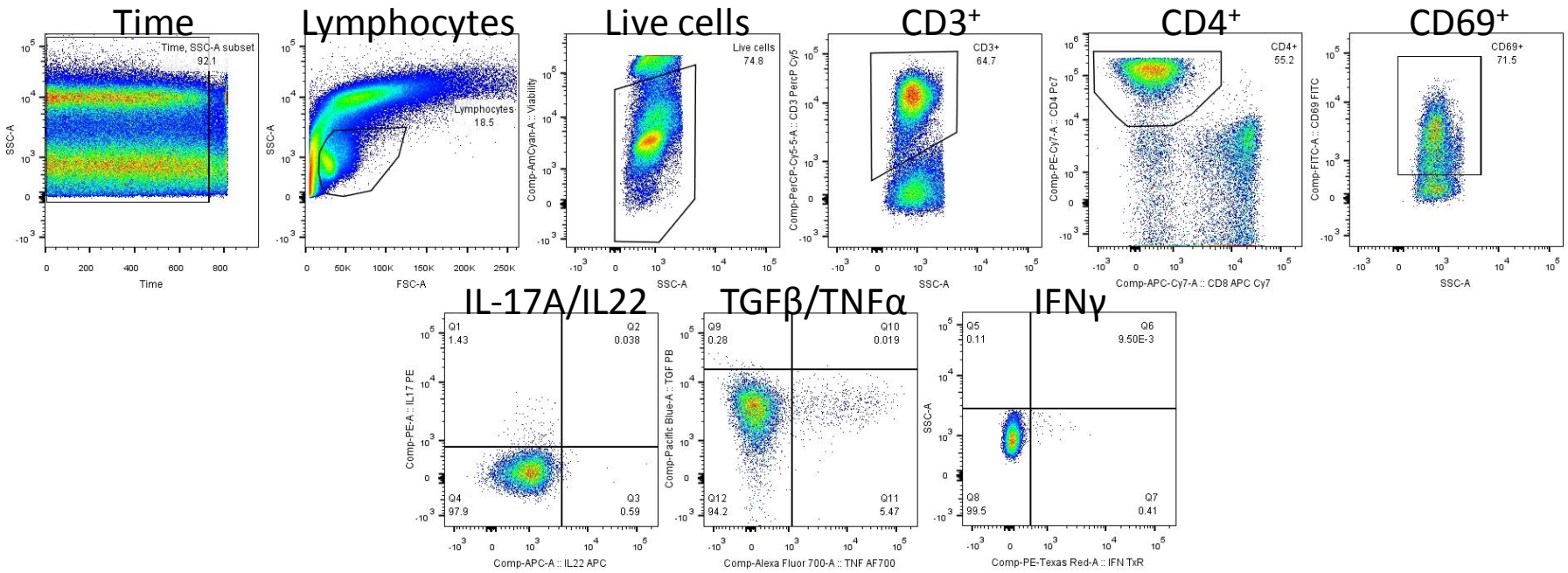


Figure 2.3 Gating strategy for detection of lung mucosal intracellular cytokine responses PspA/Rx1 and derived fragments were used in antigenic stimulation of immunological responses *ex vivo*. Multi-parameter flow cytometry was employed to compare intracellular cytokine responses between constructs. Surface receptors were labelled to distinguish $CD3^+$, $CD4^+$, $CD8^+$ and $CD69^+$ cell populations. Intracellular cytokine staining of IL-17A, IL-22, TNFα, TGFβ and IFNγ was used to compare percentage of cells expressing cytokines after stimulation with each candidate fragment. FSC – forward scatter (measure of cell size); SSC – side scatter (measure of cell granularity/ internal complexity).

CHAPTER 3

Humoral PspA-specific responses to *S. pneumoniae*

3 Humoral PspA-specific responses to *S. pneumoniae*

3.1 Introduction

Antibodies against the Pneumococcal surface protein A (PspA) have been shown as cross-reactive, cross-functional and cross-protective in mice (Moreno *et al*., 2010, Miyaji *et al*., 2002). Murine studies have spearheaded our understanding of PspA as a pneumococcal vaccine candidate, although with an emphasis on protection from invasive disease. Studies dissecting PspA's role in carriage, generation of mucosal immune responses, or implications for human pneumococcal immunity include demonstration of intranasal immunisation with PspA protecting against both carriage and invasive disease with a heterologous pneumococcal strain (Wu *et al*., 1997). Another study demonstrated that human serum PspA antibodies passively protect moribund mice infected with pneumococci expressing heterologous PspAs (Briles *et al*., 2000a).

The acquisition of the state of nasopharyngeal pneumococcal carriage is a pre-requisite for disease susceptibility. Studying carriage is important to understanding pneumococcal disease as it functions as a reservoir for *S. pneumoniae*, and so plays a role in its transmission. Therefore, dissecting the role of anti-PspA IgG responses in the prevention of carriage and its modulation of mucosal immunity is worth investigation. Limited studies have been conducted in humans on this front, but include observations of an increase in serum PspA antibodies following pneumococcal inoculation in individuals that acquired carriage (McCool *et al*., 2002), and an increase in naturally acquired serum PspA antibodies with age and therefore exposure (Rapola *et al*., 2000).

Using the platform of Experimental Human Pneumococcal Carriage (EHPC), we aimed to further current understanding of the role of serum antibodies and investigate PspA-specific family and clade cross-reactivity, associating with susceptibility to experimental carriage and re-acquisition of carriage following natural colonisation. Specifically, our research questions were:

1. Does colonisation protect against re-acquisition of carriage with heterologous PspA family-expressing *S. pneumoniae*?
2. Does pneumococcal exposure alter IgG levels to heterologous PspA families?
3. Does pneumococcal exposure modulate IgG binding to *S. pneumoniae* expressing heterologous PspA families?

This knowledge will allow for further understanding of PspA's ability to mediate carriage and affect mucosal immunity.

3.2 Materials and Methods

3.2.1 Volunteer recruitment

Healthy adult volunteers from the dose-ranging study (PspA sequencing and antibody level data) were stratified into two groups: Cohort A, negative for carriage pre-inoculation, and Cohort B, naturally colonised with *S. pneumoniae* on presentation. Both groups were inoculated with a single intranasal dose of *S. pneumoniae* serotype 6B, PspA clade and family 1 (strain BHN418), and monitored for nasopharyngeal carriage by nasal wash and classical microbiology as previously described (Section 2.3.2).

Healthy adult volunteers from the EHPC-PCV clinical trial (serum IgG purification and binding experiments), were stratified according to resultant carriage status following inoculation with a single intranasal dose of *S. pneumoniae* strain BHN418, and monitored for nasopharyngeal carriage as previously described (Section 2.3.1).

3.2.2 Detection of anti-pneumococcal antigen immunoglobulin

Anti-pneumococcal protein antibody levels were determined as previously described (Ferreira *et al*., 2013). Briefly, NUNC 96-well plates (Thermo Scientific (UK)) were coated overnight with 1 µg/mL of capture antigen (PspAs 1 – 5: from pneumococcal strains 435/96, 371/00, 259/98, 255/00 and 122/02 respectively; PspA2 strain Rx1 and daughter fragments 1 – 7: fragment

length 1011, 300, 300, 300, 300, 300 and 243 base pairs respectively) (Figure 1.5) (Vadesilho *et al*., 2014); blocked with 1% phosphate buffered saline-bovine serum albumin (PBS-BSA) to prevent non-specific binding (Sigma), samples incubated at a starting dilution of 1:80, and bound antibodies detected using anti-human IgG alkaline phosphatase (Sigma), 5 mg para-Nitrophenylphosphate (PNPP) (Sigma) and absorbance measured at 405 nm. The standard sample was a reference pooled human serum source of known anti-PspA concentration, kindly provided by Professor D. Briles (University of Alabama, USA). Anti-pneumococcal protein IgG levels were measured using a FLUOstar Omega microplate reader (BMG Labtech, UK), and analysis conducted with the MARS Data Analysis software v. 1.20 (BMG Labtech).

3.2.3 Statistical analysis

Graphical and statistical analyses were performed using GraphPad Prism version 5.0 (California, USA). The Fisher's exact test was used to determine the exact probability of a role of natural isolate PspA type in protection from re-acquisition of carriage. One-way ANOVA with Tukey's (parametric) or Dunn's (non-parametric) post-hoc testing for multiple comparisons and unpaired t-tests were performed on log transformed antibody levels to compare PspA antigen immunogenicity. An alpha level of 0.05 was used for all statistical tests (two-tailed). Flow cytometry data were analysed using FlowJo software version 7.6.5 (Treestar, Oregon, USA).

3.2.4 Pneumococcal DNA extraction, PspA clade typing and 6B-specific qPCR

Pneumococcal DNA was extracted from strains isolated from natural carriers by a modification of the QIAGEN DNA mini kit, and genomic DNA amplified as previously described (Carvalho *et al*., 2007). For PspA clade typing, genomic DNA was amplified with primers LSM12A (5' TAG CTC GAG ACC ATG ATC TTA GGG GCT GGT TT 3') and LSM9A (5' TAG TTA TCT AGA TTT TGG TGC AGG AGC TGG 3') (Invitrogen), based on primers previously described by McDaniel *et al*. (1994). Amplified DNA was cloned into a pGEM-T Easy vector (Promega, Madison, USA) and sequenced by Sanger sequencing using Universal M13F and Universal M13R primers (Invitrogen).

For 6B-specific qPCR, genomic DNA was amplified with primer wciP(cpsS) (5' GCTAGAGATGGTTCCTTCAGTTGAT/CATACTCTAGTGCAAACTTTGCAAAAT 3') and Taqman minor groove binder (MGB) probe St6B VIC (5' ACTGTCTCATGATAATT 3') using the Mx3005P system (Stratagene) as previously described by (Tarrago *et al*., 2008). Data were analysed using the instrument's software. A negative-extraction control (parallel extraction of sample buffer), a negative control (serotype 6A), two positive controls (serotype 6B), and two extractions of the sample isolate, were amplified. A cycle threshold (CT) value of > 35 was considered positive.

3.2.5 Total IgG purification from serum

Serum from ten individuals inoculated with *S. pneumoniae* serotype 6B-PspA1 was pooled. A 1:1 ratio of Protein G sepharose (GEHealthcare, Uppsala, Sweden) to pooled serum was used; the sepharose column was packed by centrifugation in a 15 mL conical centrifuge tube for 8 minutes at 900 x *g* (all centrifugation conditions the same), washed and equilibrated in two resin-bed volumes of binding buffer [20mM sodium phosphate, pH 7.0: 11.54 mL dibasic Na_2HPO_4 (in house) + 8.46 mL monobasic NaH_2PO_4 + 180 mL distilled H_2O] by re-suspending in binding buffer and centrifugation (baseline wash).

Serum was applied to the sepharose column at a 1:1 ratio, and mixed on an end-over-end rotator at room temperature for 2 hours to promote sample antibody binding. Unbound antibody was removed by four cycles of washing and centrifugation in binding buffer, or until absorbance of the wash fraction approached that of the baseline wash (at 405 nm), demonstrating absence of free antibody.

Bound IgG was released from the sepharose column by addition of a two-bed resin volume of elution buffer [0.1M Glycine-HCl, pH 2.7 (in-house)] and gentle mixing on an end-over-end rotator for 10 minutes followed by centrifugation. To ensure complete IgG elution and a concentrated recovery, elution buffer addition-mix-centrifugation steps were repeated using a one-bed resin volume. To neutralise the pH of the IgG elution fraction, 1M Tris-HCl, pH 9.0 (in house) was added to reach a pH of approximately 7.0.

3.2.6 Antigen-specific IgG purification from serum

A CNBr-activated sepharose column 4B (GEHealthcare) was prepared by re-suspension of 1 part lyophilised sepharose (mg) in 5 parts 1mM HCl (in-house) (mL), followed by four cycles of re-suspension in 1mM HCl (vortexing and centrifugation at 3,500 rpm for 20 seconds). The protein ligand to be column-coupled (CRM-197, PspA/Rx1 or Fragment 4) was dissolved in a ratio of 1 mg ligand: 1.5 mL coupling buffer [0.1M $NaHCO_3$, pH 8.3 (containing 0.5M NaCl) (in-house)], and bound to the column by mixing on an end-over-end rotator at 4°C overnight. Excess ligand was removed by centrifugation and three cycles of washing in coupling buffer and remaining active CNBr-groups blocked by addition of 0.1M Tris-HCl pH 8.0 followed by incubation for 2 hours at room temperature. The column was washed with 3 cycles of alternating pH to ensure no free ligand remained ionically bound to the immobilised ligand: acetate buffer solution pH 4.0 and 0.1M Tris-HCl pH 8.0 were used, both solutions containing 0.5M $NaCl_2$ as to minimise protein-protein adsorption and formation of protein aggregates.

The serum sample was then added to the column, and incubated with rotation for 2 hours at room temperature, with unbound antibody removed by centrifugation. The column was washed with excess phosphate buffered solution (PBS) (in-house), and bound IgG extracted by addition of elution buffer: 50mM glycine, pH 2.8 (in-house) and incubation for 2 minutes at room temperature. Elution fractions were neutralised to pH 7.0 with 0.5 M Tris buffer (in-house).

3.2.7 Visualisation of IgG by dot blot and SDS-PAGE

Total IgG wash and elution fractions were spotted onto a nitrocellulose membrane in 4 µL volumes. To prevent non-specific binding, the membrane was incubated with 5% bovine serum albumin (BSA) in Tris-Buffered Saline and 0.1% Tween 20 (TBS-T) (in house) for 45 minutes. The membrane was washed in TBST-T for 10 minutes, thrice over. Bound IgG was detected with a 1:5000 dilution of α-human IgG alkaline phosphatase (Sigma) in TBS-T 2.5% BSA for 2 hours. The membrane was washed three times in TBST-T for 10 minutes and developed using a BCIP-NBT kit (Promega) until the colorimetric reaction became visible.

Samples were run on a 10% gradient SDS gel (in-house) for IgG visualisation and assessment of sample purity. Protein bands were visualised with Coomassie blue stain.

3.2.8 Pneumococcal growth and antibody binding assays

Four *S. pneumoniae* strains were employed in antibody binding assays: un-encapsulated pneumococcal Rx1 strains expressing native PspA2 (strain STR R1 – streptomycin resistant), Janus-cassette constructed PspA knock out Rx1 Δ*pspA*, and subsequent recombinant strains Rx1 *pspA1* (St435/96) and Rx1 *pspA4* (St255/00) (Miyaji *et al*., 2015).

Pneumococcal strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) to OD_{600} 0.4 at 37°C in 5% carbon dioxide. Bacteria were concentrated 10-fold, and employed in antibody binding assays as previously described (Moreno *et al*., 2012) with modifications. Namely, the use of purified heat-inactivated IgG from a pool of 10 individuals in place of total serum for each test condition (carriage negative/positive before/after pneumococcal inoculation), and anti-human IgG FITC (Sigma) as the detection antibody.

To account for the occasional variation in distribution of pneumococcal population size and therefore detection of IgG binding, geometric mean fluorescence intensity (GMFI) of the FITC positive population was computed. Subsequent fold changes (FC) in GMFI were calculated, either in relation to controls (for optimisation assays) or inoculation time-point (ratio of post: pre IgG binding).

3.3 Results

3.3.1 Pneumococcal carriage and re-acquisition of *S. pneumoniae* expressing homologous and heterologous PspA families

We investigated whether the PspA type expressed by *S. pneumoniae* natural carriage strains associated with cross-protection from re-acquisition with *S. pneumoniae* serotype 6B expressing PspA clade 1 (6B-PspA1). We molecularly typed the PspA clade of isolates recovered from volunteers naturally colonised with different pneumococcal strains (Cohort B), and following clearance of their colonisation episode inoculated volunteers with 6B-PspA1, monitoring whether they re-acquired carriage (Table 3.1).

Table 3.1 Natural carriers PspA type and carriage re-acquisition following pneumococcal inoculation

Volunteer number	Natural carriage serogroup	PspA classification		Carriage re-acquired?	Re-colonisation rate
		Family	Clade		
1	33	1	1	Yes	1/4 (25%)
2	33	1	1	No	
3	3	1	1	No	
4	6	1	2	No	
5	3	2	3	No	3/4 (75%)
6	19	2	3	Yes	
7	15	2	4	Yes	
8	33	3	6	Yes	

S. pneumoniae isolates from volunteers who presented with natural carriage had PspA molecularly typed. After clearance of their colonisation episode, volunteers were inoculated with *S. pneumoniae* 6B-PspA1 and carriage re-acquisition monitored. Volunteers previously colonised with a PspA family 1 pneumococcus had a non-significant 67% risk reduction of re-acquiring carriage with a pneumococcus expressing a homologous family PspA (Fisher's exact test, $p = 0.49$, RR 0.33). The corresponding odds ratio was 0.11.

Amongst individuals previously colonised with *S. pneumoniae* expressing a homologous family PspA to that of the 6B-PspA1 inoculation strain (clades 1 and 2), we observed a 25% rate of re-colonisation after inoculation (n = 1 out of 4). However, in those previously colonised

with a non-family 1 PspA (clades 3 - 6), we recorded a 75% rate of re-colonisation (n = 3 out of 4).

We noted a natural carriage isolate typed as serogroup 6 for volunteer number 4: to investigate whether protection observed was serotype-specific, we performed a 6B-specific capsular qPCR. No CT value was measured, qualifying the isolate as a non-serotype 6B (Figure 3.1).

Therefore, we observed a 67% reduction in risk of re-acquiring carriage with *S. pneumoniae* expressing a homologous family PspA (Risk Ratio = 0.33) in individuals previously colonised with a PspA family 1 pneumococcus (corresponding Odds Ratio = 0.11). This result was not statistically significant (Fisher's exact test, $p = 0.49$) and to this end we note the limited sample size of this dataset (n = 8). However, taking the expected carriage rate of 50% on inoculation with *S. pneumoniae* 6B-PspA1 in a population of unknown carriage history (Gritzfeld *et al*., 2013), a halving to 25% was indicative of a potential role of homologous family PspA immunity in protection from carriage re-acquisition.

To investigate whether the observed protective potential of PspA type associated with serum IgG levels, we measured IgG levels to homologous and heterologous PspAs in carriage negative and positive healthy adult volunteer serum.

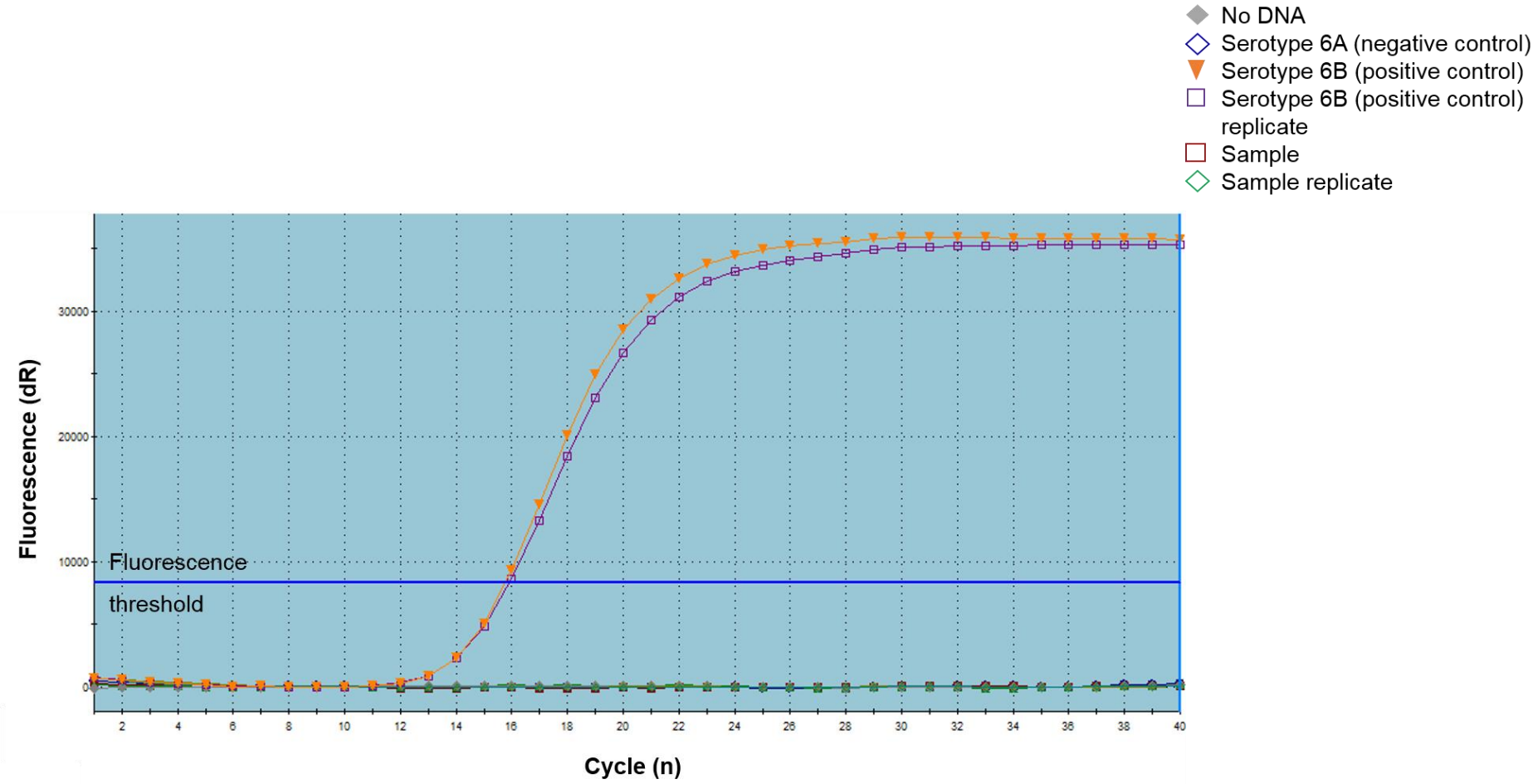


Figure 3.1 Serotype 6B-specific qPCR amplification plot. A serotype 6B-specific qPCR was conducted to identify whether volunteer 4's serogroup 6 natural carriage isolate was positive or negative for serotype B. Blue horizontal line, fluorescence detection threshold; grey triangle, no DNA control; blue diamond, serotype 6A negative control; orange triangle and purple square, serotype 6B positive controls; red square and green diamond, natural carriage isolate DNA.

3.3.2 Pneumococcal exposure and IgG levels to homologous and heterologous PspA families

We have previously reported pneumococcal carriage in healthy adults to be an immunising event generating serum IgG to PspA clades of the same family. Inoculation with 6B-PspA1, leads to increased IgG levels to PspA clade 2 (PspA2, family 1, strain UAB055) but not clade 3 (PspA3, family 2, strain UAB099) (Ferreira *et al.*, 2013).

We investigated whether exposure to *S. pneumoniae* 6B-PspA1 modulated heterologous family PspA IgG level to other family 2 clades. We also investigated whether *S. pneumoniae* 6B-PspA1 exposure altered homologous family, cross-clade PspA serum IgG reactivity. We measured anti-PspA IgG levels against PspA clades 1 to 5, in serum samples of volunteers uncolonised at baseline (Cohort A) pre- and post-inoculation with 6B-PspA1.

Study participant data were stratified by subsequent carriage status, and pre-inoculation IgG levels against PspAs 1 to 5 measured by ELISA (Figure 3.2a). Serum IgG levels to PspAs 1 to 5 varied irrespective of carriage status, significantly so in the carriage negative cohort (carriage negative, n = 16, $p < 0.01$; carriage positive, n = 12, $p = 0.19$) (One-way ANOVA with Tukey's multiple comparisons). Irrespective of carriage status (carriage negative: (c-), carriage positive: (c+)), IgG levels to PspAs 1 and 5 were high [mean value ± standard deviation - PspA1: 2.62 ± 0.70 (c-), 2.61 ± 0.46 (c+); PspA5: 2.44 ± 0.27 (c-), 2.69 ± 0.37 (c+)], and lowest to PspAs 3 and 4 [PspA3: 2.20 ± 0.26 (c-), 2.33 ± 0.34 (c+); PspA 4: 2.07 ± 0.46 (c-), 2.33 ± 0.34 (c+)]. The difference between carriage negative volunteer anti-PspA1 and PspA4 IgG levels was statistically significant on post-hoc testing (mean difference 0.55 [95% CI of difference: 0.12 to 0.98]). Although pre-inoculation PspA1 levels were elevated in carriage negative volunteers compared to levels of other PspAs, this did not differ from those who were carriage positive (difference between means: 0.01 ± 0.23 [95% CI: -0.48 to 0.49] $p = 0.98$) (unpaired t-test). Similarly, no difference was observed between carriage negative and positive volunteers IgG levels to PspA2 ($p = 0.10$), PspA3 ($p = 0.15$), PspA4 ($p = 0.11$) or PspA5 ($p = 0.07$). These results suggest that neither pre-inoculation homologous nor heterologous PspA-specific IgG protect against *S. pneumoniae* carriage. The ratio of post: pre-inoculation anti-PspA serum IgG levels was calculated for each PspA clade (Figure 3.2b). There was a universal increase in IgG levels irrespective of carriage status or PspA clade (ratio > 1).

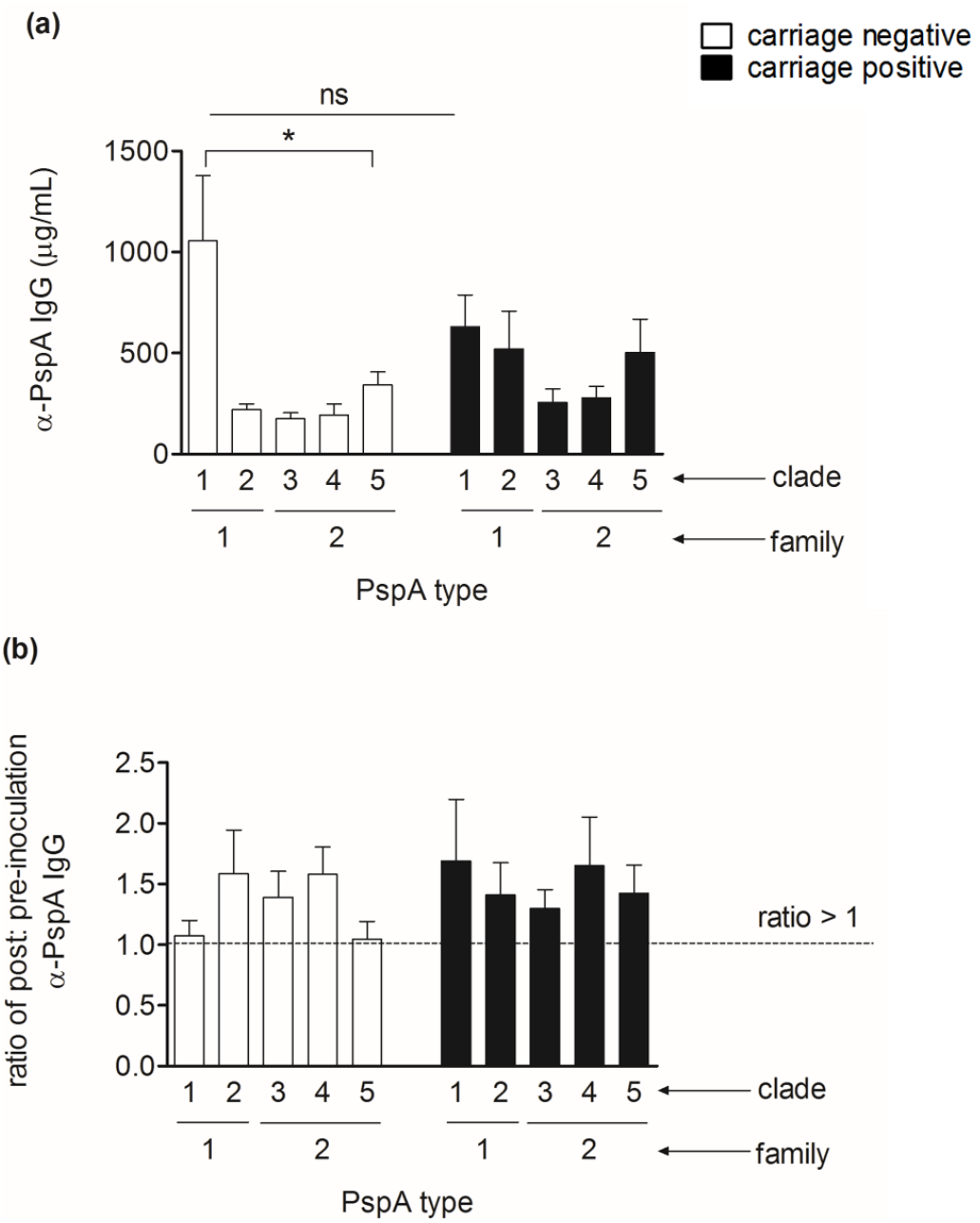


Figure 3.2 Serum IgG levels to PspA clades 1 – 5 in individuals negative and positive for experimental carriage. Antibody levels were determined by ELISA using serum from volunteers negative (n = 16, open bars) and positive (n = 12, filled bars) for experimental carriage with *S. pneumoniae* serotype 6B expressing PspA1 (6B-PspA1). (a) IgG levels against PspAs 1-5, 5 days before 6B-PspA1 inoculation (b) Ratio of PspA IgG levels before and after inoculation with 6B-PspA1. Samples obtained 14 days following inoculation were assayed for IgG levels to PspA, and the proportional fold difference plotted. *$p < 0.05$, One-way ANOVA with Tukey's post-hoc testing for multiple comparisons intra-group analysis; unpaired t-test cross-group comparisons on log transformed antibody titres.

However, this was not statistically significant for neither carriage negative (p = 0.34), nor carriage positive (p = 1.00) volunteer groups (One-way ANOVA with Dunn's multiple comparisons). Although carriage positive volunteers mounted a higher PspA1-specific IgG response on inoculation compared with carriage negative volunteers, this difference was not statistically significant (-0.62 ± 0.47 [-1.59 to 0.36] p = 0.20, unpaired t-test).

Pneumococcal exposure therefore modulated PspA family IgG levels in both carriage negative and positive volunteers: *S. pneumoniae* inoculation increased PspA-specific IgG levels to homologous and heterologous PspAs although this was not statistically significant. Furthermore, pre-inoculation IgG levels did not associate with protection from carriage.

To investigate whether cross-reactive antibody level reflected in functional activity, we employed antibody binding assays.

3.3.3 Purity of pooled serum IgG extraction

Immunoglobulin G is the predominant antibody in human serum with a high antigen specificity (Vidarsson *et al*., 2014). To assess whether increases detected in levels of cross-reactive IgG reflected in functional activity, we purified antibody from a serum pool of ten carriage negative and ten carriage positive volunteers per group:

- carriage negative volunteers pre-inoculation
- carriage negative volunteers post-inoculation
- carriage positive volunteers pre-inoculation
- carriage positive volunteers post-inoculation

Figure 3.3 illustrates detection of IgG extracted in two columns (y-axis), and probed for in column washes 2 – 5 and elution fractions 1 & 2 (x-axis). Sample IgG was released in elution 2 (dark purple). The negative control (NC) of sample buffer was free of IgG, and positive control (PC) of originating pooled serum positive for IgG.

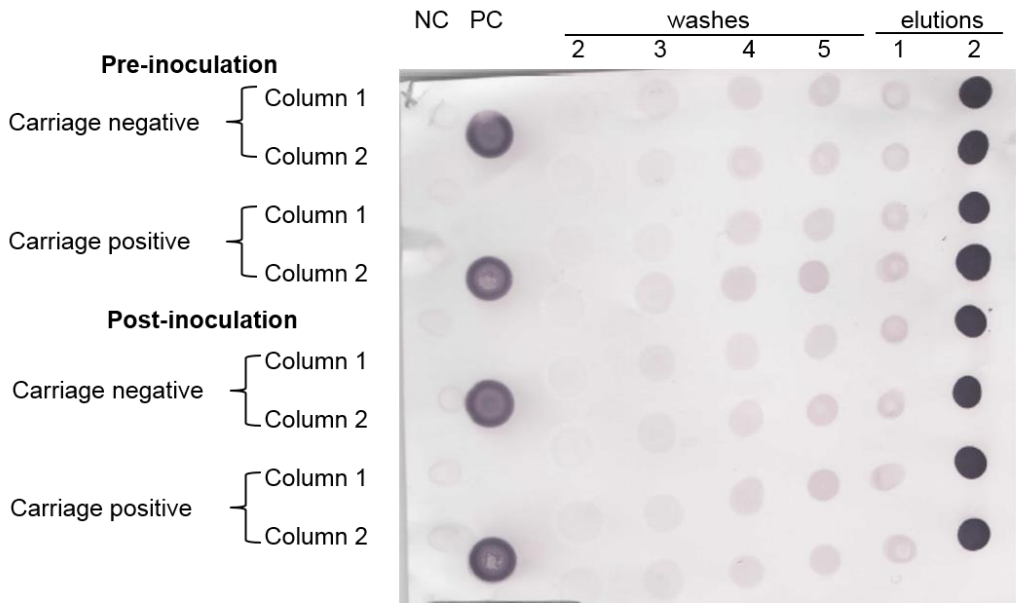


Figure 3.3 Total IgG purification dot blot to detect elution fraction. Purification conducted in two stages (Columns 1 & 2). Negative control (NC) – sample buffer; Positive control (PC) – originating pooled serum. Colorimetric reaction denotes IgG presence.

Eluted IgG was assessed for purity of sample by SDS gel electrophoresis. Three clear bands were evident in each sample: a 25 kDa IgG light chain, 50 kDa IgG heavy chain, and 66.5 kDa human serum albumin band (Figure 3.4).

Pooled serum IgG was extracted from each volunteer group and time point. Elutions were of high purity and used for downstream extraction of protein-specific IgG.

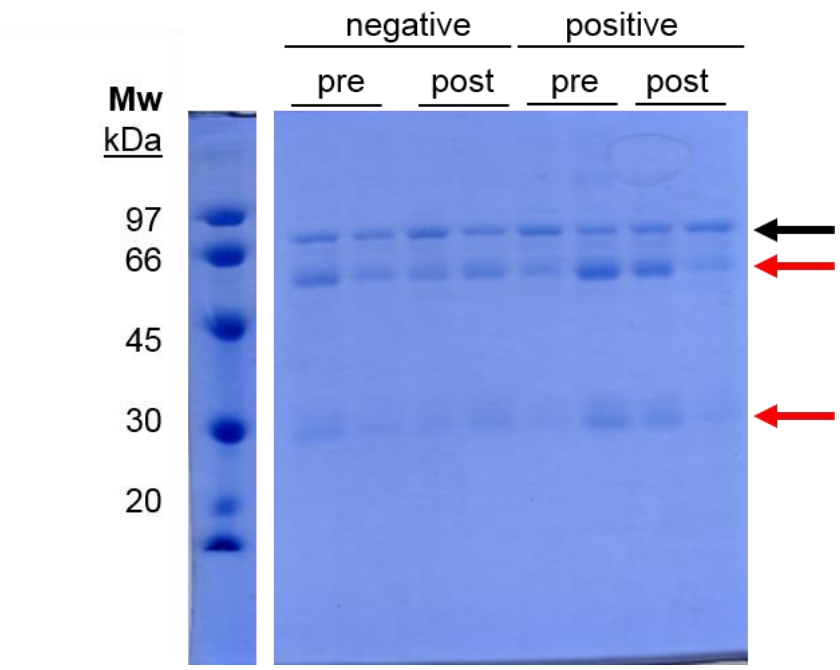


Figure 3.4 SDS-PAGE of IgG elution fractions to assess sample purity. IgG bands (red arrows) – heavy chains (50 kDa), light chains (25 kDa). Human serum albumin (black arrow) - 66.5 kDa. Duplicate pre- and post-inoculation samples correlate IgG purification columns 1 and 2 respectively. The white line between lanes 1 and 2 indicates where the gel was spliced to remove irrelevant lanes from the original gel for simplification of represented data.

3.3.4 Protein-specific IgG extraction from purified serum IgG

To investigate PspA-specific IgG pneumococcal binding, we attempted to extract these antibodies from the purified IgG fraction. Protein CRM-197 was adopted as a positive control as it is a carrier protein for multiple routine immunisations this cohort of volunteers would have received (e.g. Meningitec®, Menjugate™). For the PspA-specific IgG extraction, the parent molecule PspA/Rx1 and Fragment 4 were selected as initial antigens to pull out IgG in carriage positive samples, as previous work had demonstrated elevated binding to *S. pneumoniae* compared to other PspA fragments on immunisation with homologous protein (Vadesilho *et al.*, 2014).

In the carriage positive volunteer cohort (Figure 3.5), purification with all three proteins CRM197, PspA/Rx1 and Fragment 4 (F4), yielded IgG in two fractions: pre-inoculation unbound IgG supernatant (black brackets) and post-inoculation protein-specific elution (red

brackets). In the carriage negative cohort (Figure 3.6), IgG was detected in pre and post-inoculation unbound IgG supernatant (black brackets).

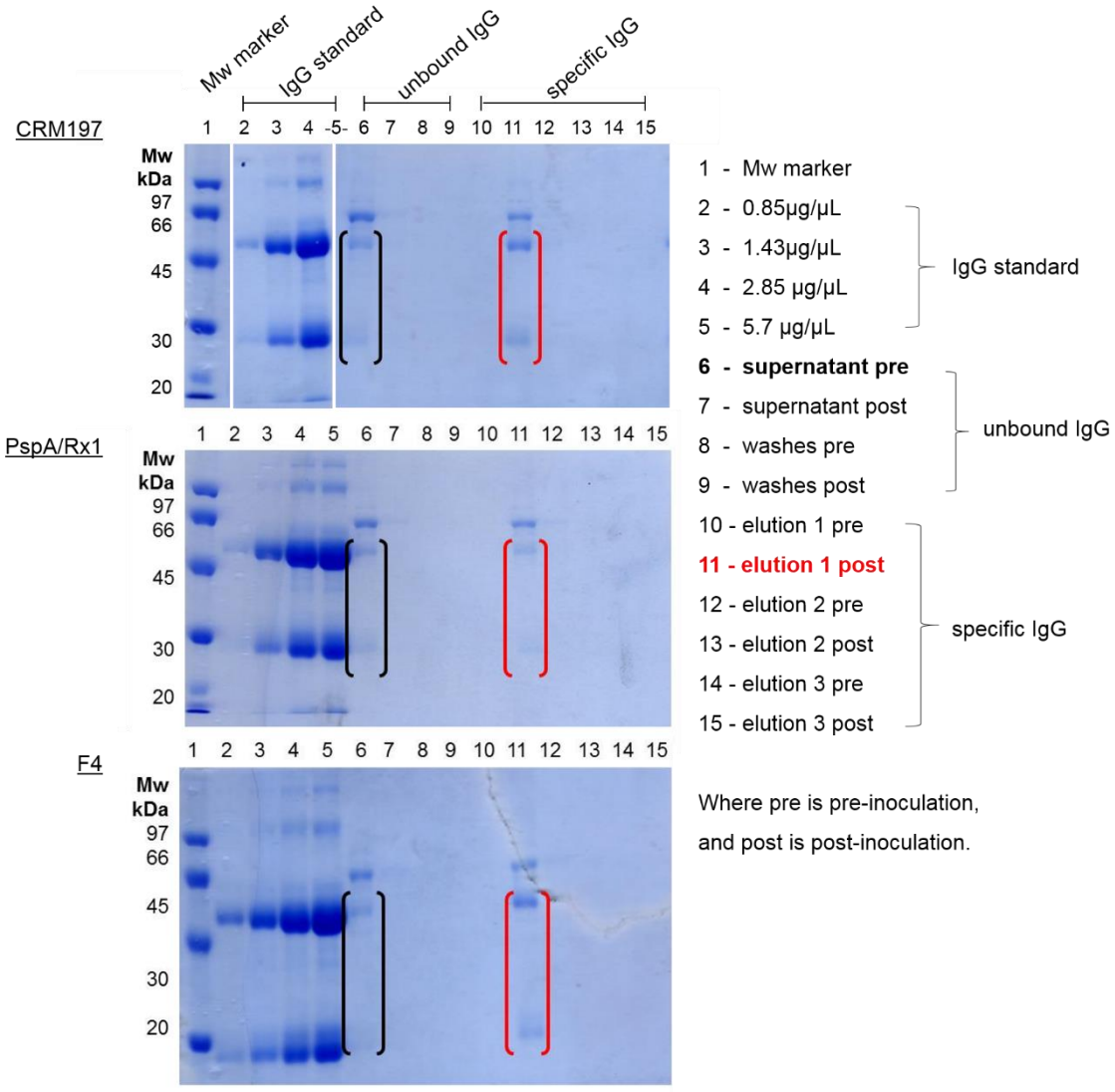


Figure 3.5 Carriage positive volunteer cohort SDS-PAGE of protein-specific IgG fractions. Protein-specific purification fractions where IgG was detected in pre-inoculation unbound IgG supernatant (black brackets) and post-inoculation CRM197, PspA/Rx1 and Fragment 4 (F4)-specific elutions (red brackets). The white lines between lanes indicate where the gel was spliced to re-order lanes from the original gel for simplification of represented data.

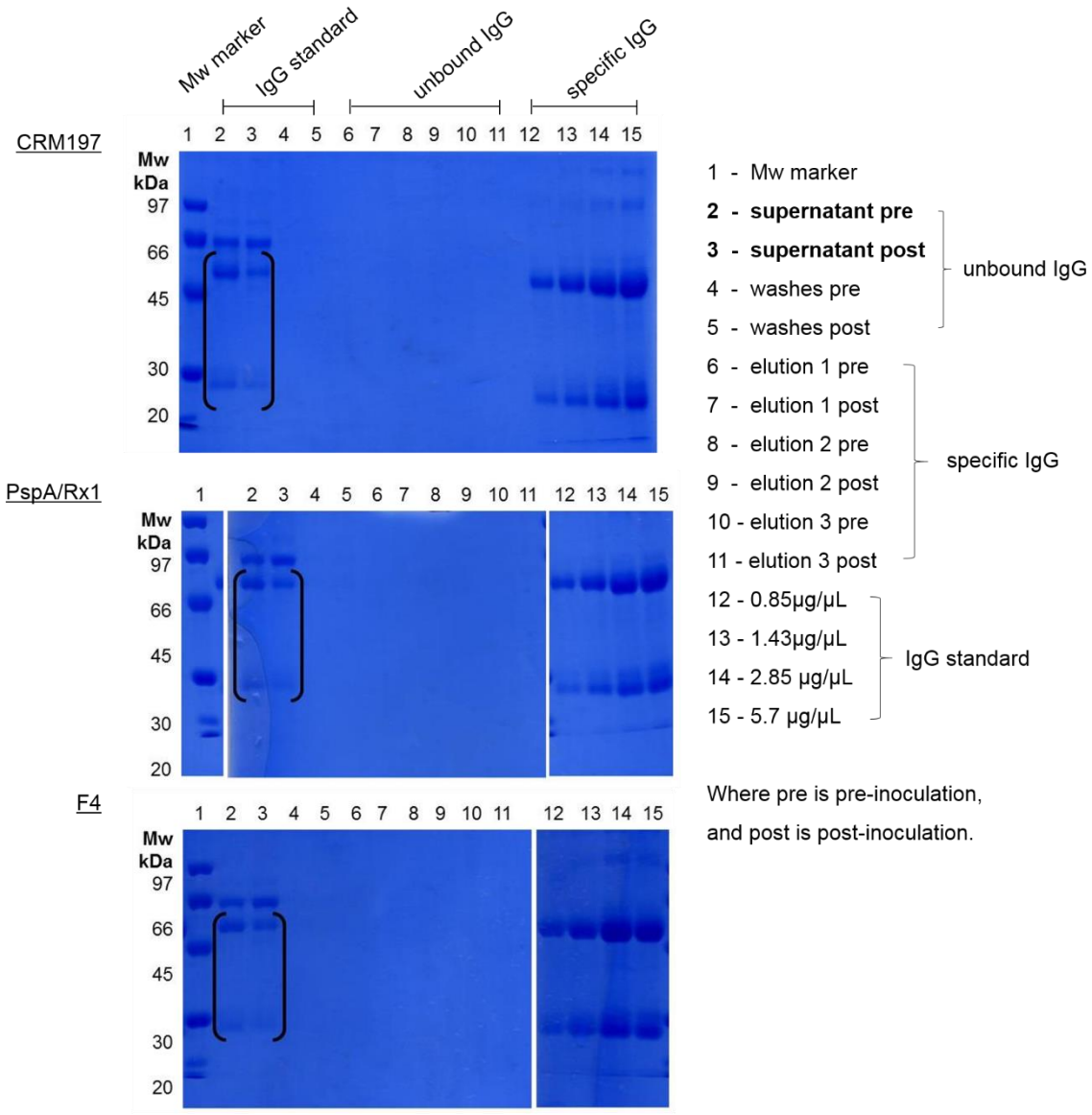


Figure 3.6 Carriage negative volunteer cohort SDS-PAGE of protein-specific IgG fractions. Protein-specific purification fractions where IgG was detected in pre- and post-inoculation unbound IgG supernatant fractions (black brackets) for CRM197, PspA/Rx1 and Fragment 4 (F4)-specific purifications. The white lines between lanes indicate where the gel was spliced to re-order lanes from the original gel for simplification of represented data.

Therefore, IgG was eluted in protein-specific fractions from carriage positive post-inoculation samples alone. Sample concentrations were calculated by densitometry analysis:

- CRM197 – 1.50 µg/µL
- PspA/Rx1 – 2.05 µg/µL
- F4 – 1.52 µg/µL

Due to achieving IgG purification from the protein-specific fraction of only 1 out of 4 experimental groups, we utilised an alternative method to interrogate PspA-specific IgG binding. Unencapsulated *S. pneumoniae* strain Rx1 and its PspA isogenic mutants were employed to investigate IgG binding in carriage negative and positive volunteer groups, in pre and post-inoculation sample pools.

3.3.5 Purified IgG binding assay optimisation

Preliminary analysis showed demonstrable binding of carriage negative volunteer pre-inoculation IgG to *S. pneumoniae pspA4* Rx1. Therefore, we titrated human anti-IgG FITC against this set-up to elucidate the optimal detection antibody concentration.

A sequential two-fold degradation in binding fluorescence was observed from a 1:10 dilution (Fold change - FC - 71.52) through to 1: 10,000 (FC 10.87) (Table 3.2). The cell count of the 1:10 dilution was 7 times less than the average of the remaining three titrations, therefore excluded. We observed an adequate separation in negative control and fluorescent peaks for the remaining dilutions, therefore we selected the practical working dilution in which the highest number of cells was recovered - 1: 1,000 FITC.

Table 3.2 Purified IgG binding detection antibody titration.

FITC dilution	Fluorescence (GMFI)	Fold change	Cell count
Unstained	460	-	-
1: 10	32900	71.52	3836
1: 100	15400	33.48	27379
1: 1000	8600	18.70	29427
1: 10000	5000	10.87	29227

GMFI = geometric mean fluorescence intensity. Fold change = stained: un-stained GMFI.

3.3.6 Effect of pneumococcal exposure on IgG binding to *S. pneumoniae* expressing homologous and heterologous PspA families at 50 µL/test

We measured IgG binding of purified IgG samples to four isogenic *S. pneumoniae* Rx1 strains in triplicate assays to investigate whether detected cross-reactive IgG levels reflected in functional activity. The strains employed were Janus-cassette constructed PspA knock out *S. pneumoniae* Rx1 ***ΔpspA***, resulting recombinant strains Rx1 *pspA1* (St435/96) and Rx1 *pspA4* (St255/00), and native Rx1 expressing PspA2 (strain STR R1) (Miyaji *et al*., 2015).

Binding of IgG was undetectable in both homologous and heterologous PspA family-expressing *S. pneumoniae* to that of the inoculation strain 6B-PspA1: *S. pneumoniae* Rx1 *pspA1*, *pspA4*, and ***ΔpspA*** fluorescence values were below that of their respective negative controls (Table 3.3). Where binding was measurable against homologous family-expressing *S. pneumoniae* Rx1 *pspA2*, pneumococcal exposure had a minimal effect on IgG binding in carriage negative volunteers (FC = 1.23), but was reduced in carriage positive volunteers (FC = 0.44) (Figure 3.7).

Due to purified IgG binding largely being undetectable above negative control levels, we concluded that the reaction may not have reached saturation due to varying purified IgG yields (Figure 3.4). Therefore, as an initial step to optimise assay conditions, we adjusted purified IgG reaction volumes to an equalised concentration.

Table 3.3 PspA binding fluorescence intensities of pre and post-inoculation purified IgG at 50 µL/test.

PspA type	Carriage status	Time point	Fluorescence (GMFI)	Fold change
1	negative	pre	-383.67	1.06
		post	-406.67	
	positive	pre	-272.67	1.01
		post	-24.33	
2	negative	pre	215.00	1.23
		post	265.33	
	positive	pre	569.00	0.44
		post	251.00	
4	negative	pre	-116.00	0.41
		post	-47.33	
	positive	pre	-131.00	0.62
		post	-81.67	
Δ	negative	pre	-462.33	0.88
		post	-408.33	
	positive	pre	-81.67	4.93
		post	-402.67	

GMFI = geometric mean fluorescence intensity. Fold change = post: pre-inoculation GMFI. Data presented graphically in Figure 3.7.

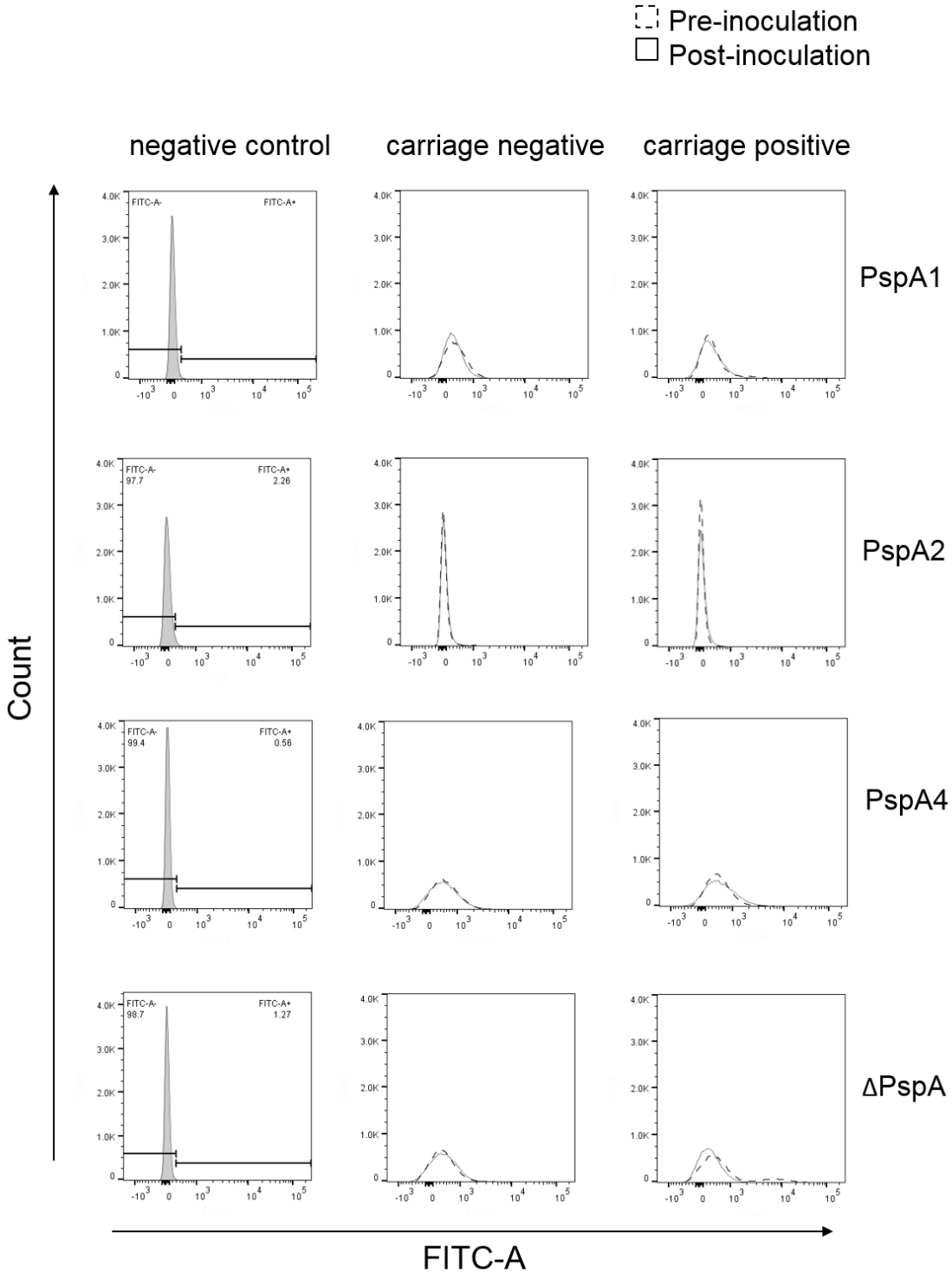


Figure 3.7 Purified IgG binding to pneumococci of varying PspA type, before and after *S. pneumoniae* 6B-PspA1 inoculation at 50 µL/test. Carriage negative and positive volunteer serum IgG binding to *S. pneumoniae* strain Rx1 expressing PspAs 1, 2, 4 or ΔPspA, before (dashed) and after (solid line) inoculation with *S. pneumoniae* serotype 6B expressing PspA1 (6B-PspA1). Binding in the absence of pooled purified IgG (grey), served as a negative control. Data presented numerically in Table 3.3.

3.3.7 Effect of pneumococcal exposure on IgG binding to *S. pneumoniae* expressing homologous and heterologous PspA families at 10 µg/test

To ensure investigation of antibody function without bias of purified IgG yields, we adjusted the concentration of samples to 10 µg/test and assayed for IgG binding to *S. pneumoniae* Rx1 strains.

Purified IgG concentrations were calculated by densitometry from SDS page gels of protein purifications. Post-inoculation IgG from carriage positive volunteers was an order of magnitude less concentrated than the other three cohorts:

- Carriage negative pre-inoculation: 0.23 µg/µL
- Carriage negative post-inoculation: 0.16 µg/µL
- Carriage positive pre-inoculation: 0.22 µg/µL
- Carriage positive post-inoculation: 0.06 µg/µL

Where purified IgG binding to *S. pneumoniae* was detectable, we observed pneumococcal exposure increased binding to pneumococci expressing each or no PspA clade. This was except for binding of purified IgG from carriage negative volunteers on binding the PspA4 variant (Figure 3.8). The greatest increase in post-inoculation binding was observed for carriage negative volunteer IgG binding to *S. pneumoniae* expressing PspA1, the homologous clade to the inoculation strain 6B-PspA1 (FC = 10.83) (Table 3.4).

Carriage positive pre-inoculation IgG binding to *S. pneumoniae* Rx1 *pspA1* and *pspA2* was an order of magnitude higher in carriage positive compared to negative volunteers, at comparable levels to *S. pneumoniae pspA4*, and diminished to *S. pneumoniae ΔpspA*. Post-inoculation IgG binding was greater in the carriage positive cohort, to all *S. pneumoniae* Rx1 strains expressing heterologous clades (Table 3.4).

Table 3.4 PspA binding fluorescence intensities of pre and post-inoculation purified IgG at 10 µg/test.

PspA type	Carriage status	Time point	Fluorescence (GMFI)	Fold change
1	negative	pre	362	10.83
		post	3919	
	positive	pre	3235	3.23
		post	10445	
2	negative	pre	633	1.79
		post	1133	
	positive	pre	1792	2.62
		post	4699	
4	negative	pre	2485	0.91
		post	2267	
	positive	pre	2663	1.71
		post	4546	
Δ	negative	pre	5971	1.19
		post	7091	
	positive	pre	3805	1.64
		post	6254	

GMFI = geometric mean fluorescence intensity. Fold change = post: pre-inoculation GMFI. Data presented graphically in Figure 3.8.

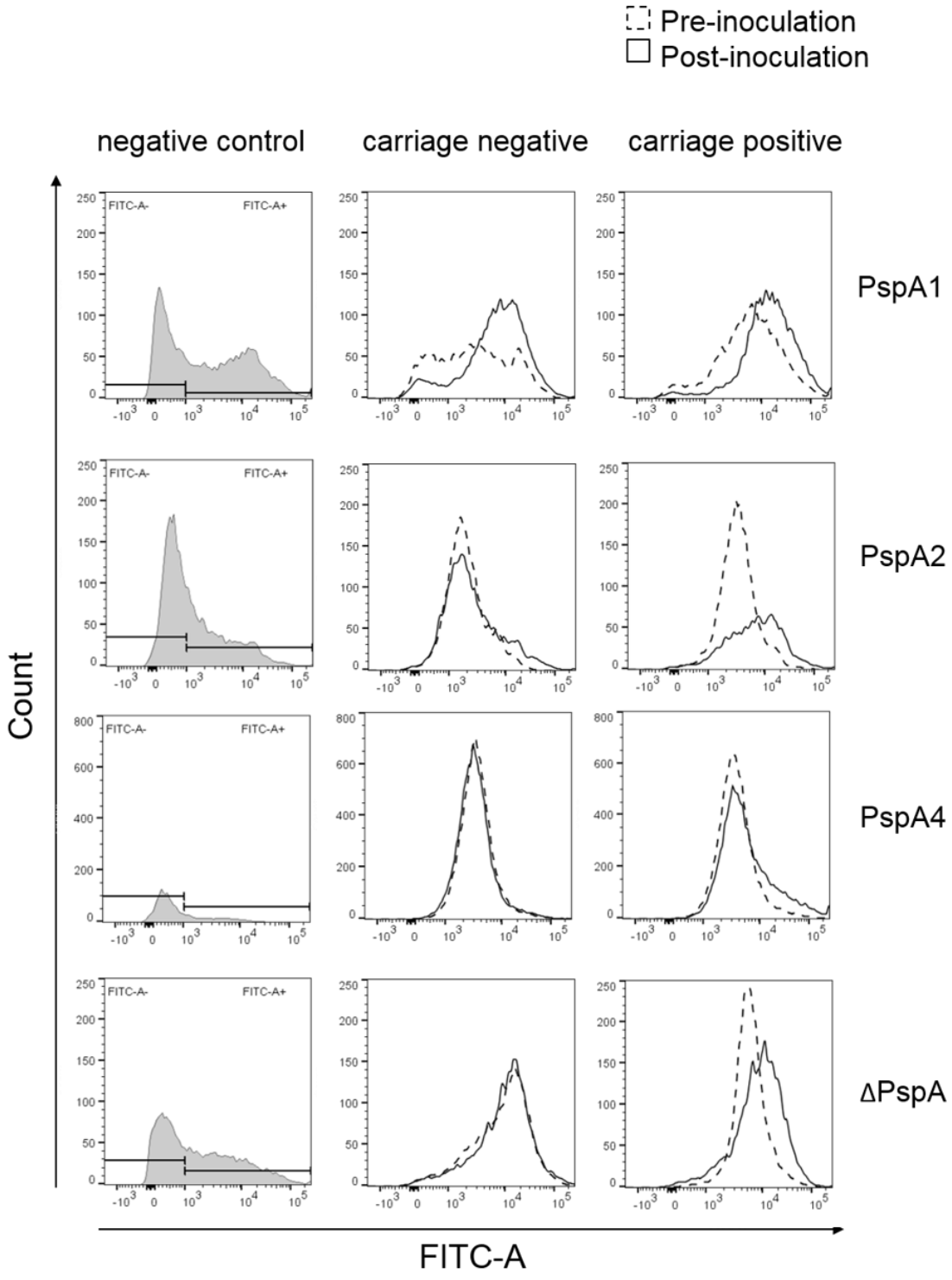


Figure 3.8 IgG binding to pneumococci of varying PspA type, before and after *S. pneumoniae* 6B-PspA1 inoculation at 10 µg/test. Carriage negative and positive volunteer serum IgG binding to S. pneumoniae strain Rx1 expressing PspAs 1, 2, 4 or ΔPspA, before (dashed) and after (solid line) inoculation with *S. pneumoniae* serotype 6B expressing PspA1 (6B-PspA1). Binding in the absence of pooled purified IgG (grey), served as a negative control. Data presented numerically in Table 3.4.

Therefore, we observed that pneumococcal exposure modulated IgG binding to *S. pneumoniae* expressing heterologous PspA families: inoculation increased purified IgG binding to *S. pneumoniae* expressing both heterologous and homologous PspA families at 10 µg/test. Carriage was immunising and PspA IgG served as a marker: carriage positive volunteer *S. pneumoniae* binding was higher compared to carriage negative volunteers. Multiple other pneumococcal proteins were observed as immunising: *S. pneumoniae ΔpspA* binding increased post-inoculation, where GMFI and hence IgG binding was notably higher than for homologous and heterologous PspA-expressing strains. We note that these data are from a single biological experiment, and pneumococcal populations grew heterogeneously.

3.4 Discussion

We observed a 50% reduction in expected colonisation rate with family-specific protection against re-colonisation in individuals previously naturally colonised: this aligned with our findings of a marked increase in clade 1-specific binding capabilities of IgG purified from carriage negative volunteers inoculated with *S. pneumoniae* 6B-PspA1. We found that pneumococcal exposure led to increased cross-reactive IgG levels to both homologous and heterologous PspA family types. However, the fold change in post: pre-inoculation IgG binding to *S. pneumoniae* with heterologous PspA families was highest in carriage positive individuals, therefore may serve as a marker of carriage and hence, disease susceptibility.

3.4.1 Questions answered: study in field context

Cross-reactivity and functionality of PspA clade 4 and 5-specific IgG has been previously reported and leads to cross-protection in a murine intranasal lethal challenge model (Moreno *et al*., 2010). Similarly, immunisation with PspA4 and intranasal lethal challenge with a heterologous family PspA2-expressing strain has demonstrated greater levels of protection than immunisation with homologous family PspA1, highlighting the cross-protective nature of PspA4 (Miyaji *et al*., 2015). We did not observe a difference in pre- and post-inoculation IgG

binding to *S. pneumoniae* Rx1 *pspA4* in a protected population. We note that this observation is based on a single biological replicate.

Our observations of protection against pneumococcal carriage re-acquisition conferred by carriage being restricted to a homologous PspA family, corroborate previous work in mice. Miyaji and colleagues (2002) demonstrated a lack of cross-protection between PspA families after PspA1 immunisation and heterologous lethal challenge in mice, despite observed cross-reactivity of PspA antibodies.

A higher cross-family post: pre-inoculation fold change in carriage positive compared to carriage negative volunteer serum IgG binding to *S. pneumoniae* expressing different PspA types, contrasted the clade-specific nature of carriage negative volunteer post: pre-inoculation fold change in serum IgG binding. From this preliminary finding, we observed an increased antibody cross-functionality conferred by nasopharyngeal colonisation, re-affirming pneumococcal carriage as immunising, and serum IgG binding as a marker of prolonged exposure and disease susceptibility in humans.

The ability to purify PspA fragment-specific IgG and assess pneumococcal binding to strains expressing various PspA clades may have provided insight into fragment-specific IgG cross-functionality, adding to our understanding of the therapeutic potential of these PspA fragments. We have previously demonstrated that older adults have reduced levels of IgG to PspA fragments when compared to younger adults. This finding was Fragment 2 (F2) and 7 (F7)-specific: F2 located in the alpha helical domain, and F7 the proline-rich region (Owugha *et al*., in preparation). This highlighted their potential as unique markers of increased susceptibility to pneumococcal infection in at risk groups. Further still, Simell *et al*. (2008) report decreased IgG titres to PspA in the elderly, with evidence of waning PspA immunity over a lifetime, in a population known to have low pneumococcal carriage rates (Almeida *et al*., 2014), and high disease susceptibility (Regev-Yochay *et al*., 2004). This strengthens the case of PspA as a probe of pneumococcal exposure, and therefore disease susceptibility.

3.4.2 Questions raised: study limitations

Our founding observations of natural carriage with pneumococci expressing homologous but not heterologous PspA families playing a potential role in protection against re-acquisition is notably limited by sample size (n = 8). However, taking our expected carriage rate of 50% in a population of unknown carriage history (Gritzfeld *et al*., 2013), a halving to 25% is suggestive of a role of homologous family PspA immunity in protection from carriage re-acquisition.

Our initial purified IgG functional binding experiments with *S. pneumoniae* strain Rx1 *pspA2* and isogenic variants *pspA1*, *pspA4* and *ΔpspA* at 50 µL serum IgG/ test, demonstrated no difference in antibody binding before and after *S. pneumoniae* inoculation. We concluded that the reaction may not have reached saturation for each of the conditions due to the varying purified IgG yields, and therefore adjusted the experiment to investigate binding at an equalised purified serum IgG concentration of 10 µg/test to separate questions of observed binding due to IgG levels versus functionality. We note as a limitation that subsequent assay results were derived from a single experiment.

Although we measured increased functionality of carriage negative volunteer IgG compared to their carriage positive counterparts on binding *S. pneumoniae* expressing PspA1, the homologous clade to inoculation strain 6B-PspA1, we note that the pneumococcal populations represented were at different growth stages. A distinct negative population would allow for better discrimination between fluorescence peaks, resolving the truest binding differences. Again, we note that this data was from a single experiment, with observations only remaining valid if reproducible.

We sought to investigate PspA fragment-specific IgG binding to *S. pneumoniae*. It has been reported that immunisation of mice with conformational epitopes of PspA2 (PspA fragments) protected mice against lethal challenge with a pneumococcus expressing a homologous clade (strain A66.1, PspA2) (Vadesilho *et al*., 2014). Fragments 4, 5 and parent protein PspA/Rx1 induced the highest protection, therefore we attempted purification of Fragment 4 and PspA/Rx1 serum IgG in addition to the immunogenic vaccine conjugate control, CRM197. We have recently demonstrated that fragments in the alpha-helical and proline-rich regions of PspA (Fragments 1, 2 and 7) displayed elevated IgG levels on nasal *S. pneumoniae* exposure

in healthy adults (Owugha *et al*., in preparation). These regions have been identified to contain protective epitopes (McDaniel *et al*., 1994, Daniels *et al*., 2010). Furthermore, Fragments 2 and 7 were shown to be uniquely elevated in younger compared to older adults, implicating them as markers of pneumococcal exposure (Owugha *et al*., in preparation): these fragments may have served as more efficient probes for pulling down PspA fragment-specific IgG. Although we could detect IgG in the protein-specific post-inoculation elution fractions from the carriage positive volunteer cohort for each protein, we were unable to detect protein-specific IgG in elution fractions for carriage negative volunteers. Therefore, to compare pneumococcal binding by carriage status, we investigated total IgG binding to *S. pneumoniae* of varying PspA type, in place of protein-specific IgG to *S. pneumoniae*.

3.4.3 Questions posed: further work and study implications

Although PspA has been studied to determine whether it is a broadly protective antigen and vaccine candidate, it is now widely accepted that for its success, formulations must include antigens across the two major PspA families (family 1 and 2). Further still, multiple pneumococcal vaccine formulations now focus on combining fragments of multiple antigens (Katsura *et al*., 2014, Lu *et al*., 2015), so investigating smaller PspA constructs is of utility for incorporation into aforementioned strategies.

We demonstrated binding of serum IgG from individuals exposed to 6B-PspA1, to *S. pneumoniae* ***ΔpspA*** from both carriage negative and positive groups, pre and post-inoculation. Fluorescence IgG binding values were largely higher for the knock out strain compared to PspA-expressing strains, despite possessing the same genetic backbone (*S. pneumoniae* Rx1). My explanatory hypothesis is that PspA molecules expressed on the surface of Rx1 strains *pspA1, pspA4* and wild type expressing PspA2, hindered IgG binding to other pneumococcal proteins. This hypothesis reflects the molecular structure of PspA where its flexible proline-rich region enables its extension through the capsule above other proteins, and the abundance of PspA expression on the pneumococcal surface resulting in a measurable cumulative effect of reduced non-PspA protein binding site access on PspA expression. This data again highlighted the involvement of other pneumococcal proteins in

humoral response to *S. pneumoniae*, and merits further investigation into protein vaccine candidates.

It is important to note that humoral immunity is solely one aspect of immune defence against pneumococcal colonisation, with PspA-specific immunity being one of multiple proteins involved. The importance of $CD4^+$ T-cells in protection from pneumococcal carriage has been demonstrated in mice (Malley *et al*., 2004), with particular emphasis placed on the role of the Th17 cell subset in recent years (Bogaert *et al*., 2009). Importantly, Trzciński and colleagues show both the $CD4^+$ T-cell mechanism of infection protection as antigen-specific (Trzciński *et al*., 2008), and anti-PspA IgG correlating to, but not be required for protection from colonisation in a murine model (Trzciński *et al*., 2005). Therefore, it is imperative to dissect both the roles of humoral and $CD4^+$ T-cell responses to PspA fragments to better understand their potential for inclusion in multiple-epitope vaccine formulations in broad protection against pneumococcal disease.

CHAPTER 4

Blood PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae*

4 Blood PspA-specific CD4$^+$ T-cell responses to *S. pneumoniae*

4.1 Introduction

Cellular immune responses to *Streptococcus pneumoniae* act to limit pneumococcal carriage density: Lu *et al*. (2008) demonstrated a role of the Th17 subset of CD4$^+$ T-cells, and subsequent neutrophil-directed pneumococcal clearance. As a high pneumococcal nasopharyngeal colonisation density has been correlated clinically with pneumonia susceptibility (Wolter *et al*., 2014), it is pertinent to investigate immunological mechanisms involved in protection against carriage acquisition and reduction of colonisation density and duration as a precursor to disease.

Single and multiple surface antigen formulations of virulence factors known to induce adaptive immunity whilst being widely expressed across pneumococcal strains, are deemed good novel vaccine candidates (Miyaji *et al*., 2013). These are necessary to overcome current vaccine limitations, particularly serotype-specificity. A leading antigen is the Pneumococcal surface protein A (PspA): however, despite PspA being conserved as a virulence factor across all known pneumococcal strains (Crain *et al*., 1990), it is heterogeneous in sequence and is classified into six clades and three families: family 1 (clades 1 and 2), family 2 (clades 3 - 5) and family 3 (clade 6) (Hollingshead *et al*., 2000). Vaccine design efforts have shifted toward alternative PspA constructs capable of inducing antibodies cross-reactive with strains expressing PspAs of the most prevalent families, 1 and 2 (Darrieux *et al*., 2007, Darrieux *et al*., 2008). Vadesilho and colleagues (2014) demonstrated protection against pneumonia in a murine model on immunisation with 100 amino acids (aa) PspA fragments. Protective epitopes were located in the N-terminal alpha helical domain (Fragment 2: 79 - 178 aa) and the clade-defining region (Fragments 4: 179 - 278 aa), eliciting 100% and 67% protection respectively. No protection was afforded on immunisation with Fragment 7 of the proline-rich region (329 - 406 aa), whereas 100% protection was elicited by the parent protein PspA/Rx1.

Protection from pneumococcal carriage acquisition has been shown in murine studies to be CD4$^+$ Th17 dependent (Malley *et al*., 2005, Bogaert *et al*., 2009) and antigen-specific (Trzciński *et al*., 2008). Wright *et al*. (2013) demonstrated the ability to measure

pneumococcal-specific $CD4^+$ T-cell responses in human volunteers. Here we investigate whether PspA (Fragments 2, 4, 7 and parent protein PspA/Rx1)-specific $CD4^+$ T-cell immune response to *S. pneumoniae* is detectable in blood, and if so, whether it associates with protection from carriage in healthy adults exposed to *S. pneumoniae* in our Experimental Human Pneumococcal Carriage (EHPC) model. Specifically, our research questions were:

1. Are antigen-specific $CD4^+$ T-cell responses to *S. pneumoniae* detectable in blood?
2. Does inoculation alter antigen-specific $CD4^+$ T-cell responses to *S. pneumoniae*?
3. Do changes to pre-existing antigen-specific $CD4^+$ T-cell responses to *S. pneumoniae* post-inoculation associate with carriage status?
4. Do post-inoculation antigen-specific $CD4^+$ T-cell responses to *S. pneumoniae* associate with colonisation intensity (area under the curve analysis of density and duration)?

4.2 Materials and Methods

4.2.1 Volunteer Recruitment and the Experimental Human Pneumococcal Carriage (EHPC) Model

Study approval was granted by the Liverpool Central NHS Research Ethics Committee [REC 12/NW/0873]. Written informed consent was obtained from each healthy adult volunteer to enrol in the EHPC-PCV study (EudraCT 2012-005141-20). Pneumococcal inoculation and subsequent detection was carried out as previously described (Gritzfeld *et al*., 2013). In short, volunteers were inoculated with a single intranasal dose of 80,000 cfu/ 100 µL per naris serotype 6B (BHN418 strain), and acquisition of carriage determined by microbiological culture of nasal wash.

4.2.2 Expression of recombinant proteins

PspA fragments originating from *S. pneumoniae* strain Rx1 (PspA/Rx1) were engineered into ampicillin-selectable and IPTG-inducible expression vector pAE (Ramos *et al*., 2004) to produce plasmids pAE-*frag 2* (300 bp), pAE-*frag 4* (300 bp), pAE-*frag 7* (243 bp) and pAE-*pspARx1* (1,011 bp) as previously described (Vadesilho *et al*., 2014). Constructs were expressed in *Escherichia coli* BL21/DE3 (Studier and Moffatt, 1986) as His-tagged PspA/Rx1 fragments, and purified from the soluble expressed fraction using Ni^{2+} affinity chromatography (Qiagen). Resultant proteins were dialysed using 7K MWCO SnakeSkin™ dialysis tubing (ThermoScientific, Rochford, (USA)) and quantified by densitometry using SDS gel electrophoresis reads and GelAnalyzer2010® software (Lazar, 2017).

4.2.3 Tissue processing and *ex vivo* stimulation

Blood samples were obtained 5 days before and 21 days after *S. pneumoniae* inoculation. Peripheral blood mononuclear cells (PBMCs) were isolated by density-mediated centrifugation using Ficoll-Paque PLUS (GEHealthcare, Uppsala (Sweden)) according to the manufacturer's instructions. PBMCs were washed twice in PBS, re-suspended in complete media [RPMI + 20 mM L-glutamine supplemented with antibiotics (penicillin 40 U/mL, streptomycin 40 µg/mL,

neomycin 80 µg/mL) and 10% Foetal Calf Serum (FCS) (Invitrogen)], and seeded into a 24-well plate in a volume of 1 mL and concentration of 1 x 10^6 PBMCs/mL Cells were left unstimulated (for background subtraction of non-specific cytokine secretion), and separately stimulated with 4 µg/mL PspA antigens (PspA/Rx1, Fragments 2, 4 and 7), and 0.5 µg/mL positive assay control staphylococcus enterotoxin B (SEB). PBMCs were incubated at 37°C, 5% CO_2 for 120 hours and in the final 16 hours of stimulation, 1 µL of a 3 mM monensin stock (BD Biosciences, San Diego (USA)) and 1 µL of a 1mg/mL brefeldin stock (BD Biosciences) were added to each well, preventing release of induced cytokines from the endoplasmic reticulum and Golgi apparatus of lymphocytes.

4.2.4 Flow Cytometry

Cells were harvested by centrifugation at 533 x *g* for 20 minutes, stained for surface phenotyping and intracellular cytokine production, and acquired on a BD LSR II flow cytometer. Viable $CD3^+CD4^+$ $CD69^+$ T-cells (hereafter described as $CD4^+$ T-cells) and IL-17A, IL-22, TNFα, IFNγ and TGFβ secretors, were identified using FlowJo software v10 (Treestar, Oregon, USA). Pestle v1.7 and SPICE v.5.2 software (Mario Roederer, Vaccine Research Centre, NIAID, NIH) were employed for background subtraction and data mining respectively.

4.2.5 Statistical Analysis

Data analysis was conducted as previously described (Section 2.4.4). Briefly, stimulus-specific cytokine secreting $CD4^+$ T-cell data underwent initial analysis by One-way ANOVA with Dunnett's post-hoc testing, calculation of geometric mean percentage cytokine-secreting $CD4^+$ T-cells and subsequent fold change (FC) relative to unstimulated control. Mean volunteer FC across all stimuli (average FC all stimuli) is presented.

Subsequent exploratory analysis employed log-transformed percentage cytokine-secreting $CD4^+$ T-cell data which followed a Gaussian distribution. Therefore, parametric tests were employed, namely t-tests both paired and unpaired, and Pearson's correlation tests. Graphical representation and statistical analyses were performed using GraphPad Prism v5.0 (California, USA). Differences were considered significant at $p < 0.05$ (two-tailed).

4.3 Results

4.3.1 Detection of PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* in healthy adults

The ability to detect whole cell pneumococcal-specific CD4⁺ T-cell responses in blood has been described (Wright *et al*., 2012). In order to harness the EHPC model to test novel serotype-independent vaccine candidates, we assessed the sensitivity of our assay to detect induction of single pneumococcal antigen-specific CD4⁺ T-cell responses to *S. pneumoniae* in blood.

We observed detectable PspA-specific CD4⁺IL-17A⁺, IL22⁺, TNFα⁺, IFNγ⁺ and TGFβ⁺ cytokine responses to *S. pneumoniae* above unstimulated levels (US) both pre- and post-pneumococcal inoculation, with average fold change measurements (all stimuli) > 1 (Table 4.1).

Pre-inoculation, the greatest measurable differences in proportion of CD4⁺ T-cells secreting cytokine in response to protein stimulation were in CD4⁺IL-17A⁺ T-cells ($p = 0.02$), TNFα⁺ ($p = 0.03$) and TGFβ⁺ ($p = 0.01$) (One-way ANOVA with Dunnett's post-hoc test, $p < 0.05$) (Figure 4.1a). Fragment 7-specific CD4⁺ T-cell induction was consistently detected at each of the measurable cytokine outputs after post-hoc corrections (mean difference [95% CI of difference]: IL-17A (-0.10 [-0.17 to -0.02]); TNFα (-0.03 [-0.05 to -0.01]); TGFβ (-0.10 [-0.18 to -0.02])). Levels of CD4⁺ T-cell secreting IL22 ($p = 0.42$) and IFNγ ($p = 0.15$) were not significantly different from their unstimulated controls.

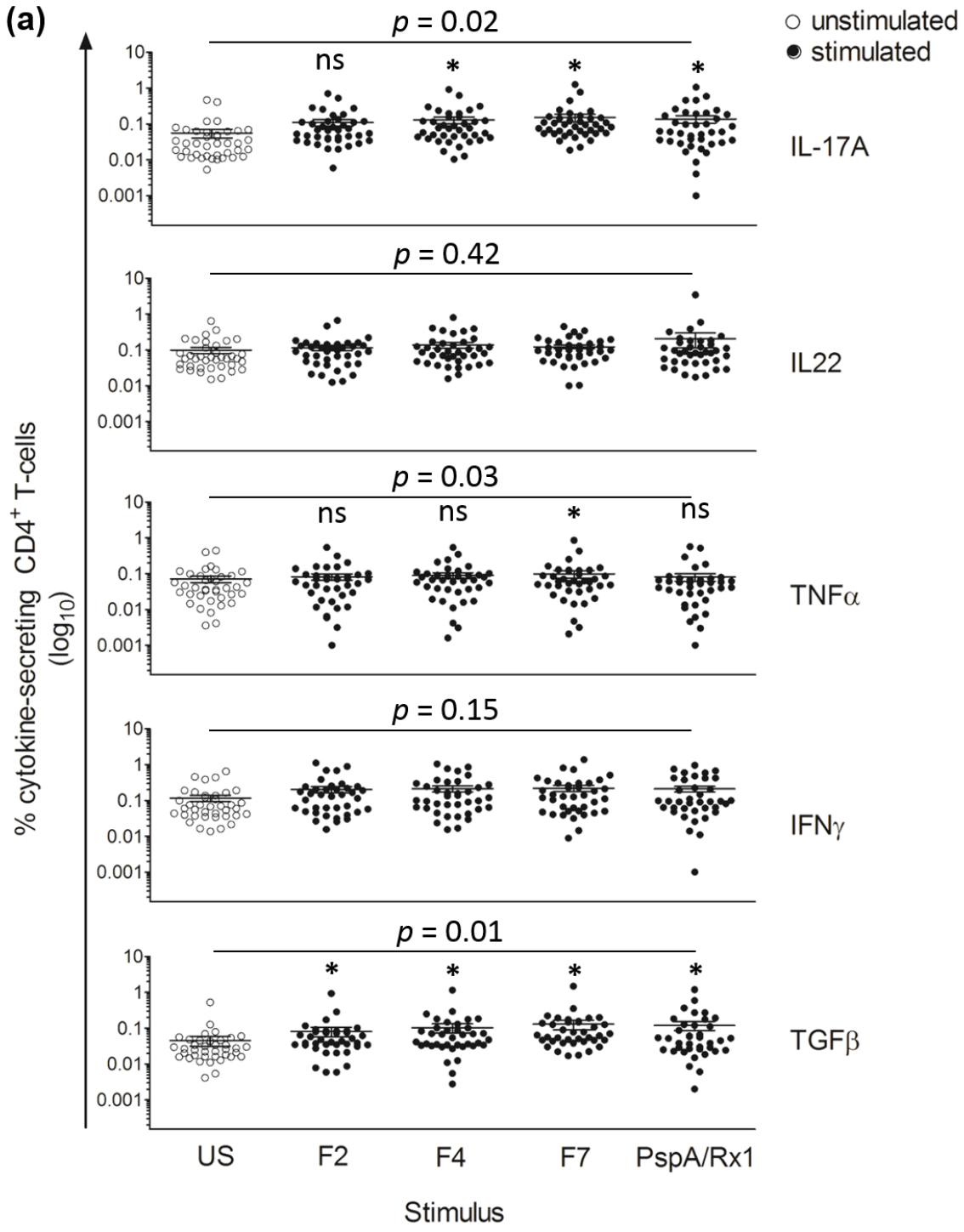
To investigate whether PspA-specific CD4⁺ T-cell responses were detectable after pneumococcal exposure, we measured post-inoculation PspA-specific CD4⁺ T-cell responses to *S. pneumoniae*. All average combined stimulus CD4⁺ T-cell responses (all stimuli) were detectable, that is above their unstimulated control (FC > 1, Table 4.1). Levels of post-inoculation PspA-specific CD4⁺ T-cells secreting TGFβ were significantly increased ($p = 0.01$) (One-way ANOVA with Dunnett's post-hoc test, $p < 0.05$). Overall protein responses were not statistically significant for IL-17A ($p = 0.06$), IL22 ($p = 0.06$), TNFα ($p = 0.73$), or IFNγ ($p = 0.06$) (Figure 4.1b).

Therefore, antigen-specific $CD4^+$ T-cell responses to *S. pneumoniae* were detectable in blood: we demonstrated the ability to detect PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* in the blood of healthy adults both pre-and post-inoculation using the EHPC platform.

Table 4.1 Fold changes in pre- and post-inoculation blood unstimulated to stimulated PspA-specific CD4⁺ T-cells.

Average percentage cytokine secreting CD4⁺ T-cells												
	Pre-inoculation						Post-inoculation					
	US	F2	F4	F7	PspA/Rx1	Average Fold Change (all stimuli)	US	F2	F4	F7	PspA/ Rx1	Average Fold Change (all stimuli)
IL-17A	0.03 [0.02 to 0.04]	0.07 [0.05 to 0.09]	0.08 [0.06 to 0.11]	0.09 [0.07 to 0.12]	0.06 [0.04 to 0.10]	**2.50** [2.13 to 2.92]	0.05 [0.03 to 0.09]	0.10 [0.06 to 0.17]	0.11 [0.07 to 0.18]	0.16 [0.10 to 0.24]	0.09 [0.05 to 0.14]	**2.09** [1.73 to 2.53]
IL22	0.07 [0.05 to 0.09]	0.08 [0.06 to 0.11]	0.09 [0.07 to 0.12]	0.09 [0.07 to 0.12]	0.09 [0.06 to 0.13]	**1.35** [1.19 to 1.53]	0.06 [0.04 to 0.09]	0.12 [0.07 to 0.18]	0.10 [0.07 to 0.14]	0.10 [0.06 to 0.16]	0.07 [0.05 to 0.11]	**1.58** [1.32 to 1.91]
TNFα	0.04 [0.03 to 0.06]	0.04 [0.03 to 0.07]	0.05 [0.03 to 0.08]	0.05 [0.04 to 0.08]	0.04 [0.03 to 0.06]	**1.14** [1.04 to 1.25]	0.05 [0.03 to 0.08]	0.07 [0.04 to 0.12]	0.06 [0.04 to 0.10]	0.08 [0.05 to 0.14]	0.07 [0.04 to 0.10]	**1.45** [1.23 to 1.72]
IFNγ	0.03 [0.02 to 0.04]	0.05 [0.03 to 0.06]	0.05 [0.04 to 0.08]	0.08 [0.06 to 0.10]	0.05 [0.03 to 0.08]	**2.05** [1.77 to 2.36]	0.03 [0.02 to 0.05]	0.07 [0.05 to 0.09]	0.06 [0.04 to 0.09]	0.08 [0.06 to 0.13]	0.05 [0.04 to 0.07]	**2.47** [1.92 to 3.18]
TGFβ	0.07 [0.05 to 0.10]	0.12 [0.08 to 0.17]	0.12 [0.09 to 0.17]	0.12 [0.09 to 0.18]	0.11 [0.07 to 0.16]	**1.59** [1.40 to 1.81]	0.08 [0.06 to 0.11]	0.17 [0.10 to 0.28]	0.15 [0.10 to 0.23]	0.19 [0.12 to 0.28]	0.15 [0.10 to 0.23]	**2.03** [1.73 to 2.38]

Average percentage cytokine secreting CD4⁺ T-cells – geometric mean [95% confidence interval of geometric mean]. US – unstimulated; F*n* – fragment *n*. Average fold change all stimuli – mean fold change from unstimulated to stimulated for F2, F4, F7 and PspA/Rx1 stimuli.


(a)
○ unstimulated
● stimulated
p = 0.02
ns
*
*
*
IL-17A
p = 0.42
IL22
p = 0.03
ns
ns
*
ns
TNFα
p = 0.15
IFNγ
p = 0.01
*
*
*
*
TGFβ
10
1
0.1
0.01
0.001
% cytokine-secreting CD4+ T-cells (log10)
US
F2
F4
F7
PspA/Rx1
Stimulus

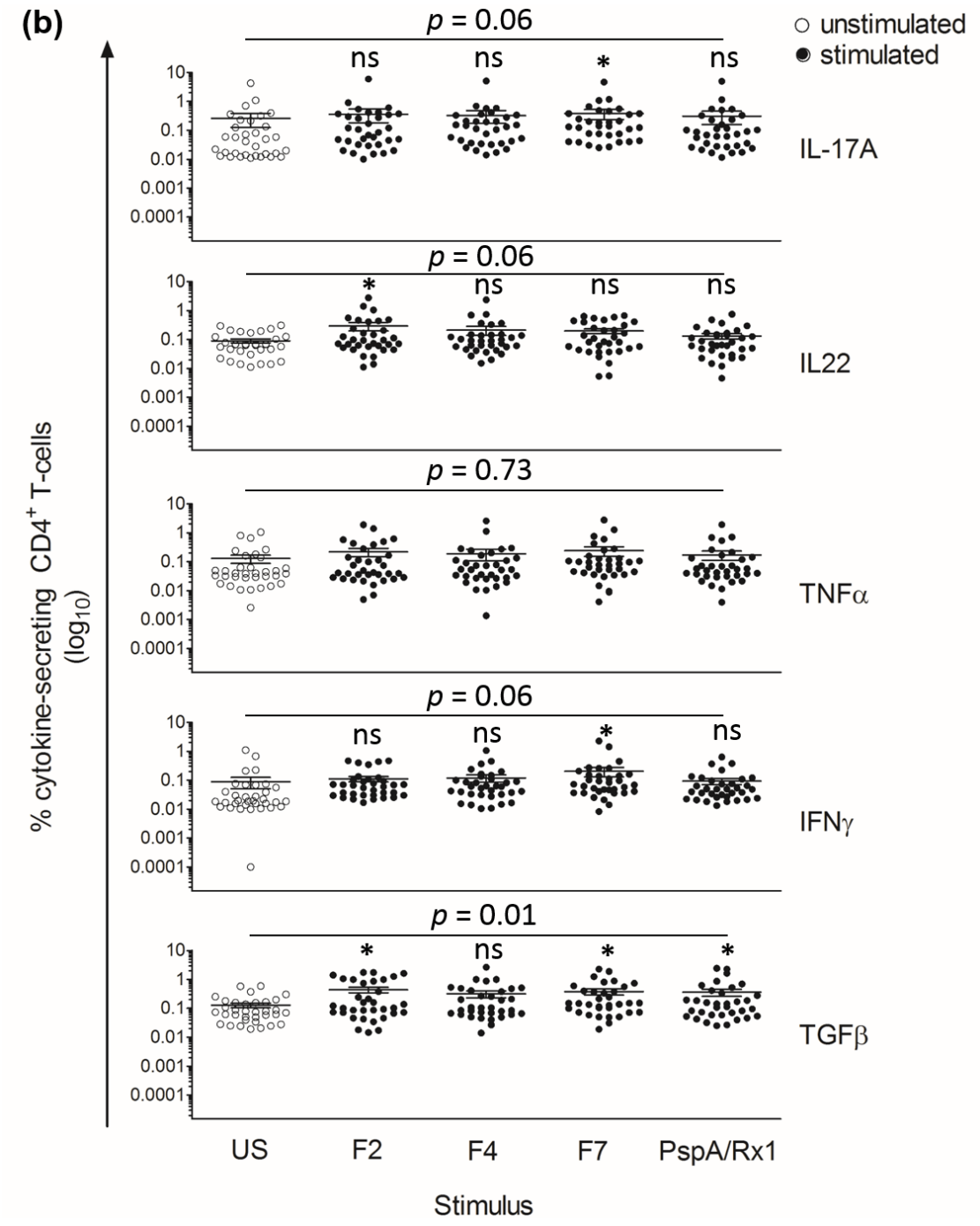


Figure 4.1 Detection of PspA-specific CD4+ T-cell responses to *S. pneumoniae* (a) Pre-inoculation and (b) Post-inoculation. Percentage cytokine-secreting CD4⁺ T-cells unstimulated (open circles) and PspA/Rx1 fragment-stimulated (closed circles). IL-17A, IL22, TNFα, IFNγ and TGFβ-secretion profiles per panel. PspA Fragments 2 (F2), 4 (F4), 7 (F7) and parent protein (PspA/Rx1) stimuli. One-way ANOVA with Dunnett's post-hoc test, * = $p < 0.05$. Horizontal bar represents mean ± SEM.

4.3.2 Post-inoculation changes to pre-existing PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* and association with carriage status

We have observed that pneumococcal carriage with *S. pneumoniae* expressing one PspA type increases levels of IgG that are cross-reactive and cross-functional to heterologous PspAs (Section 3.3.7). To investigate whether the boosting effect of carriage on humoral immunity was seen in altered $CD4^+$ T-cell immunity, we compared pre- and post-inoculation proportions of PspA-specific $CD4^+$ T-cells to *S. pneumoniae* in carriage negative and positive individuals (Figure 4.2). We note that overall volunteer post-inoculation PspA-induced responses (carriage negative and positive volunteers) were not significantly different to pre-existing responses for TNFα (FC > 1, $p > 0.05$), whereas they were significantly increased for IL-17A, IL22, IFNγ and TGFβ (Section 4.3.1).

In carriage positive individuals, we observed a boost in post-inoculation PspA-specific $CD4^+TNF\alpha^+$ T-cell response compared to pre-inoculation (Figure 4.2c). This was significant for F2 (difference in means [95% CI] -0.98 [-1.85 to -0.12] $p = 0.03$) and F7-specific (-0.49 [-0.91 to -0.07] $p = 0.03$) $CD4^+TNF\alpha^+$ responses (Paired t-test) (Section 4.3.1). Pre-existing PspA-specific $CD4^+IL\text{-}17A^+$, $IL22^+$, $IFN\gamma^+$ and $TGF\beta^+$ T-cell responses to *S. pneumoniae* remained unchanged post-inoculation in both carriage negative and positive volunteers ($p > 0.05$) (Figure 4.2a, b, d and e).

Therefore, pneumococcal inoculation altered antigen-specific $CD4^+$ T-cell responses to *S. pneumoniae*: post-inoculation Fragment 2 and 7-specific $CD4^+TNF\alpha^+$ inflammatory responses were boosted in carriage positive volunteers alone, highlighting these responses as markers of pneumococcal carriage.

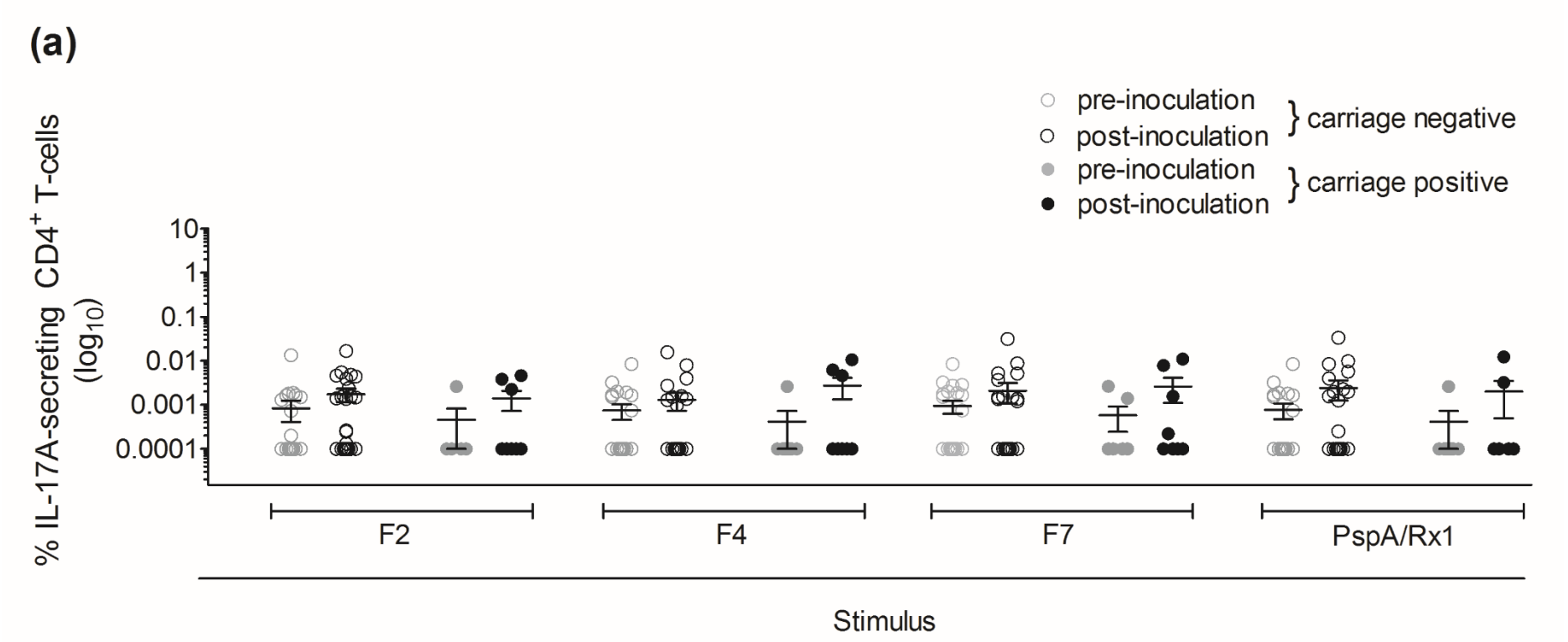


Figure 4.2 (a) Post-inoculation changes in PspA-specific CD4⁺ IL-17A T-cell responses to *S. pneumoniae* in carriage negative and positive volunteers. Background-corrected (unstimulated discounted) CD4⁺ T-cell responses in carriage negative (open circles) and positive (closed circles) volunteers. PspA Fragments 2 (F2), 4 (F4), 7 (F7) and parent protein (PspA/Rx1) stimuli. Paired t-test on log transformed data, all data * $p > 0.05$. Horizontal bar represents mean ± SEM.

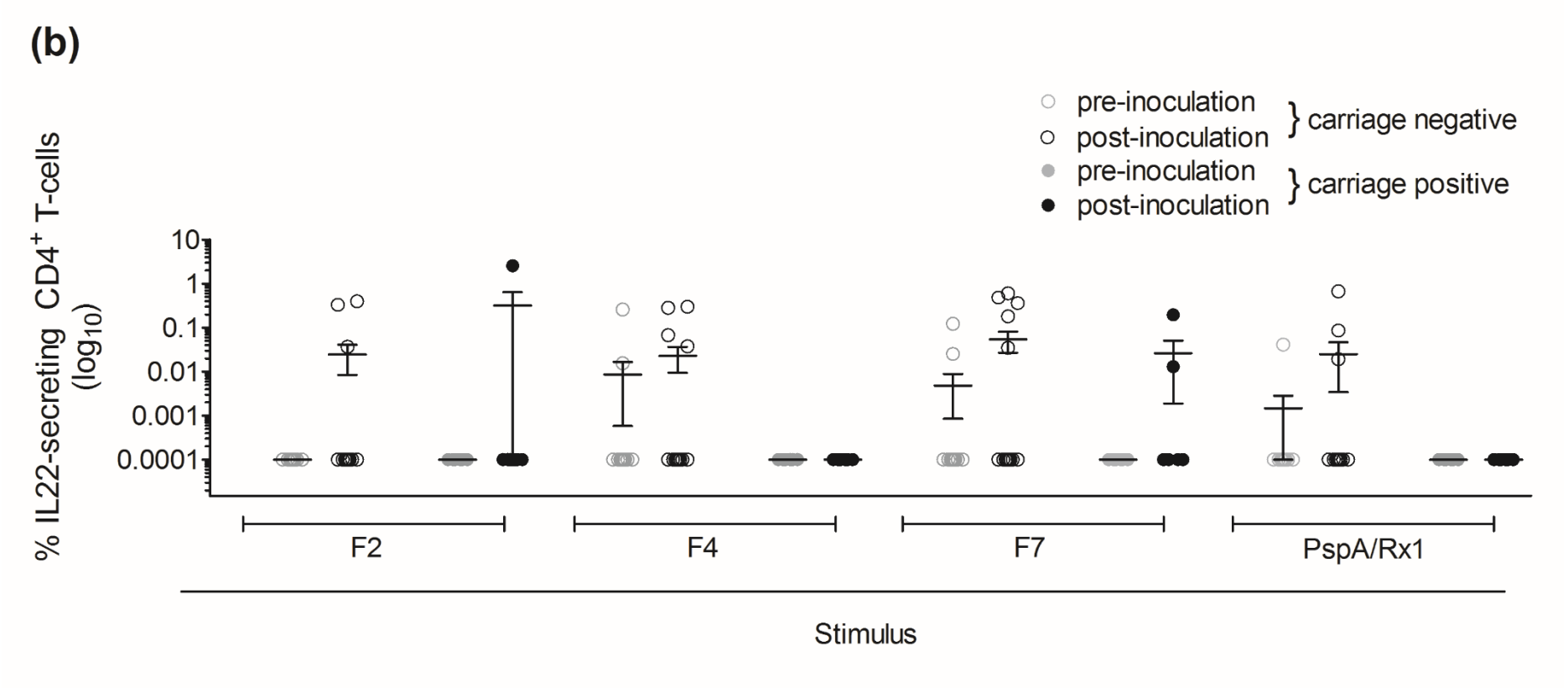


Figure 4.2 (b) Post-inoculation changes in PspA-specific CD4⁺ IL22 T-cell responses to *S. pneumoniae* in carriage negative and positive volunteers. Background-corrected (unstimulated discounted) CD4⁺ T-cell responses in carriage negative (open circles) and positive (closed circles) volunteers. PspA Fragments 2 (F2), 4 (F4), 7 (F7) and parent protein (PspA/Rx1) stimuli. Paired t-test on log transformed data, all data * $p > 0.05$. Horizontal bar represents mean ± SEM.

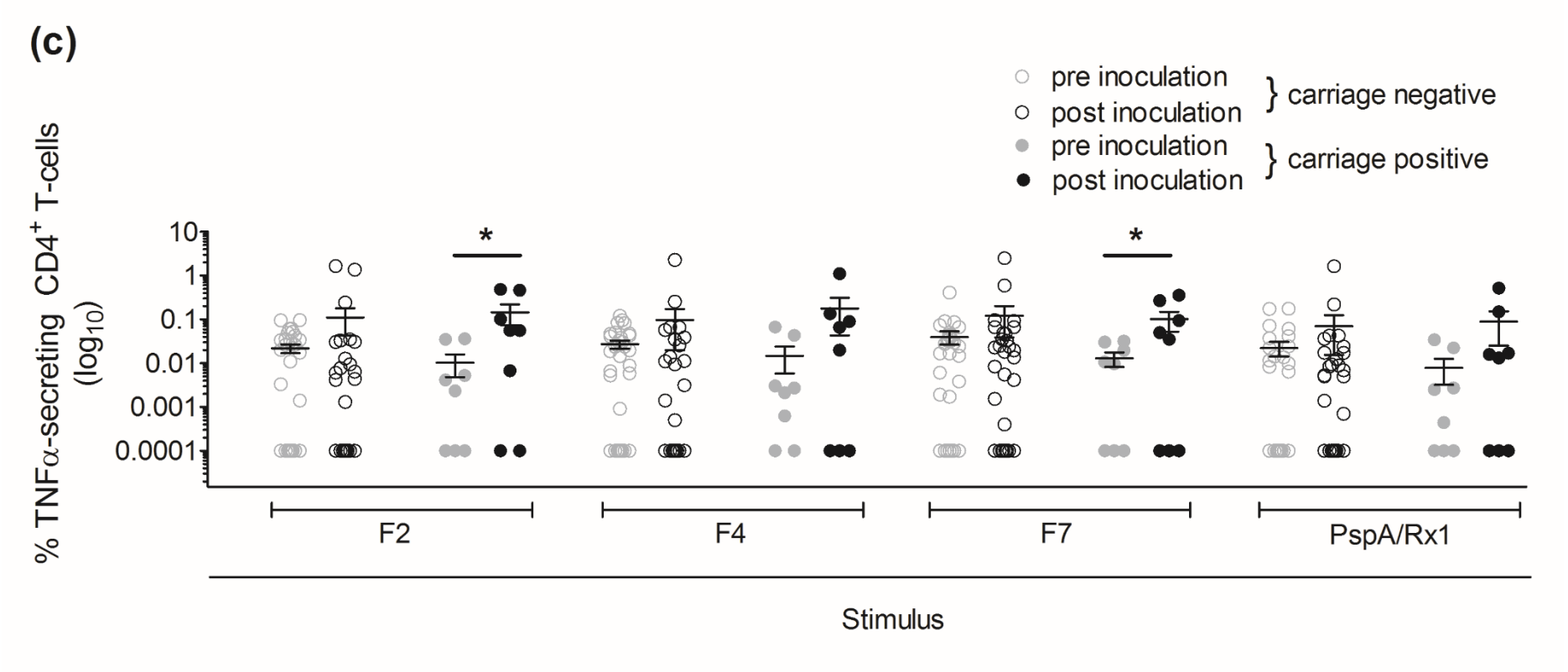


Figure 4.2 (c) Post-inoculation changes in PspA-specific CD4⁺ TNFα T-cell responses to *S. pneumoniae* in carriage negative and positive volunteers. Background-corrected (unstimulated discounted) CD4⁺ T-cell responses in carriage negative (open circles) and positive (closed circles) volunteers. PspA Fragments 2 (F2), 4 (F4), 7 (F7) and parent protein (PspA/Rx1) stimuli. Paired t-test on log transformed data, * $p < 0.05$. Horizontal bar represents mean ± SEM.

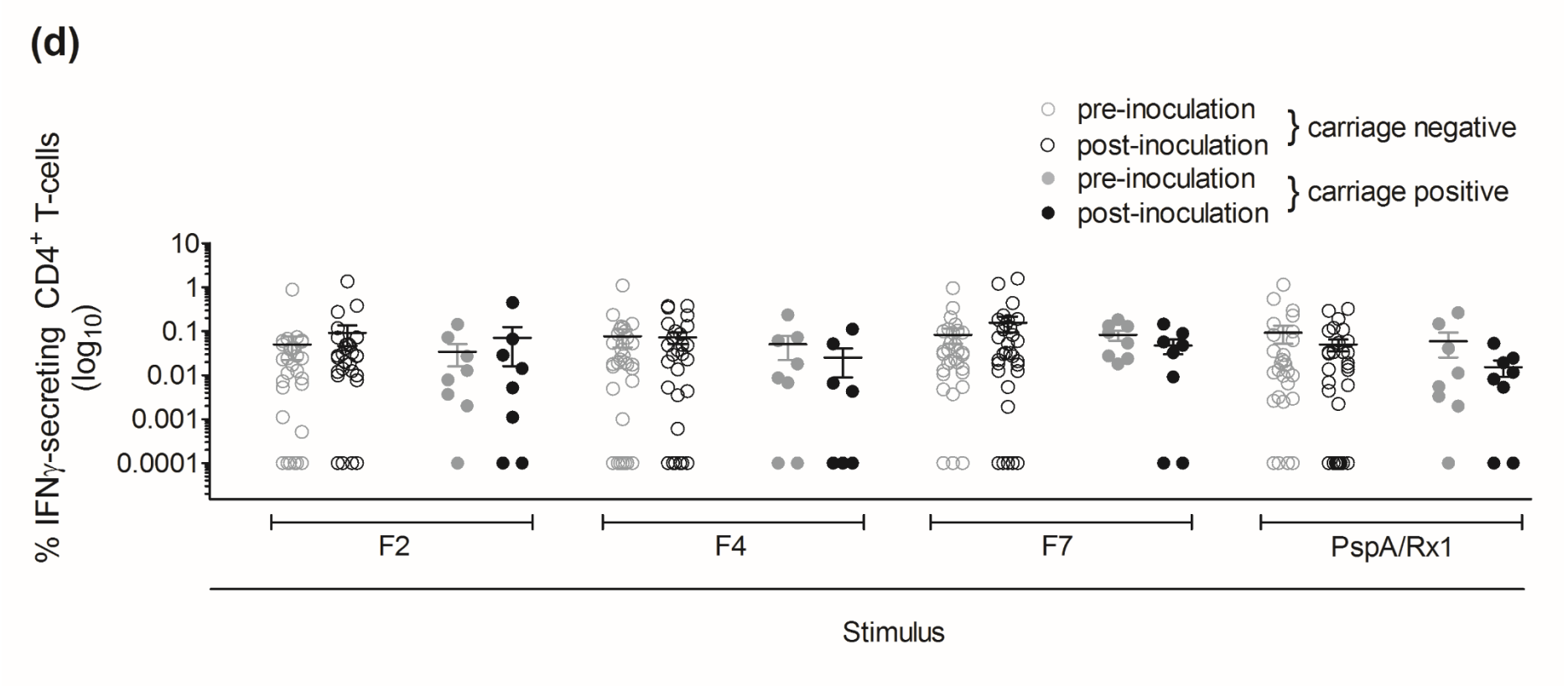


Figure 4.2 (d) Post-inoculation changes in PspA-specific CD4⁺ IFNγ T-cell responses to *S. pneumoniae* in carriage negative and positive volunteers. Background-corrected (unstimulated discounted) CD4⁺ T-cell responses in carriage negative (open circles) and positive (closed circles) volunteers. PspA Fragments 2 (F2), 4 (F4), 7 (F7) and parent protein (PspA/Rx1) stimuli. Paired t-test on log transformed data, all data * $p > 0.05$. Horizontal bar represents mean ± SEM.

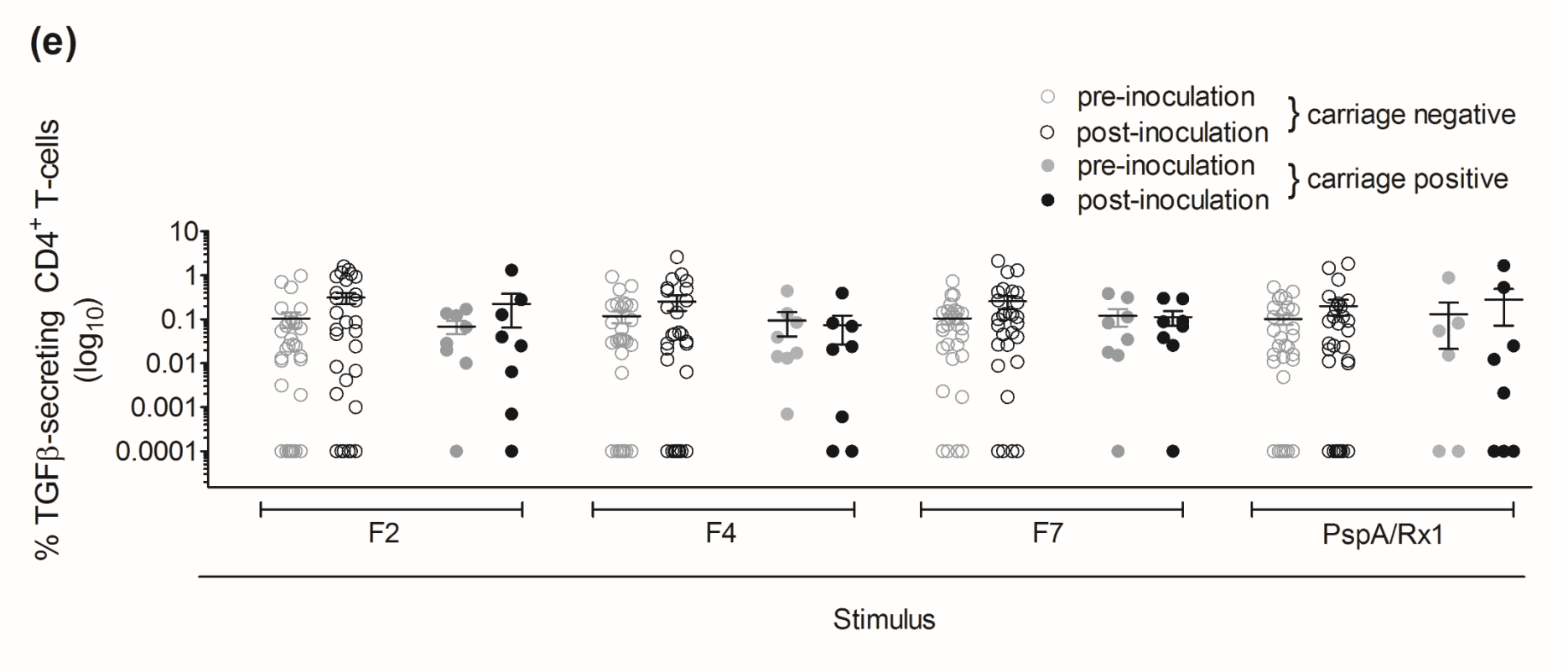


Figure 4.2 (e) Post-inoculation changes in PspA-specific CD4⁺ TGFβ T-cell responses to *S. pneumoniae* in carriage negative and positive volunteers. Background-corrected (unstimulated discounted) CD4⁺ T-cell responses in carriage negative (open circles) and positive (closed circles) volunteers. PspA Fragments 2 (F2), 4 (F4), 7 (F7) and parent protein (PspA/Rx1) stimuli. Paired t-test on log transformed data, all data * $p > 0.05$. Horizontal bar represents mean ± SEM.

4.3.3 Colonisation intensity and correlation with PspA-specific CD4⁺ T-cell responses to *S. pneumoniae*

To investigate whether there was an association between PspA-specific CD4⁺ T-cell cytokine responses to *S. pneumoniae* and a continuous measure of pneumococcal carriage, colonisation intensity, we calculated a compound measure of carriage density and duration (area under the curve analysis of density of pneumococcus recovered in nasal wash (log_{10}) and duration of colonisation in days) (Table 4.2). This measure of extent of colonisation was adopted to conduct a correlation analysis with baseline-corrected post-inoculation PspA-specific CD4⁺ T-cell cytokine-responses to *S. pneumoniae* in carriage positive volunteers for which there was reliable microbiology and flow cytometry data: two volunteers were excluded from this analysis (n = 6). Where combined total volunteer post-inoculation responses (IL-17A, IL22, IFNγ and TGFβ) were significantly greater than their unstimulated control, cytokines are emboldened (Figure 4.4).

Table 4.2 Colonisation intensity of carriage positive volunteers

Duration (days)	Volunteer					
	1	2	3	4	5	6
	Density (log_{10}cfu/mL)					
Day 2	2.15	3.39	2.03	3.36	3.90	2.35
Day 7	1.81	4.51	0.66	3.77	2.43	3.00
Day 17	-	2.01	1.07	2.56	3.54	1.46
Day 21	3.78	-	0.34	1.33	-	0.40
	Colonisation intensity (log_{10}cfu.days/mL^{-1})					
	49.10	42.52	16.44	53.60	36.70	35.50

Colonisation intensity = Area Under the Curve (AUC) analysis of density and duration of pneumococcal carriage episode.

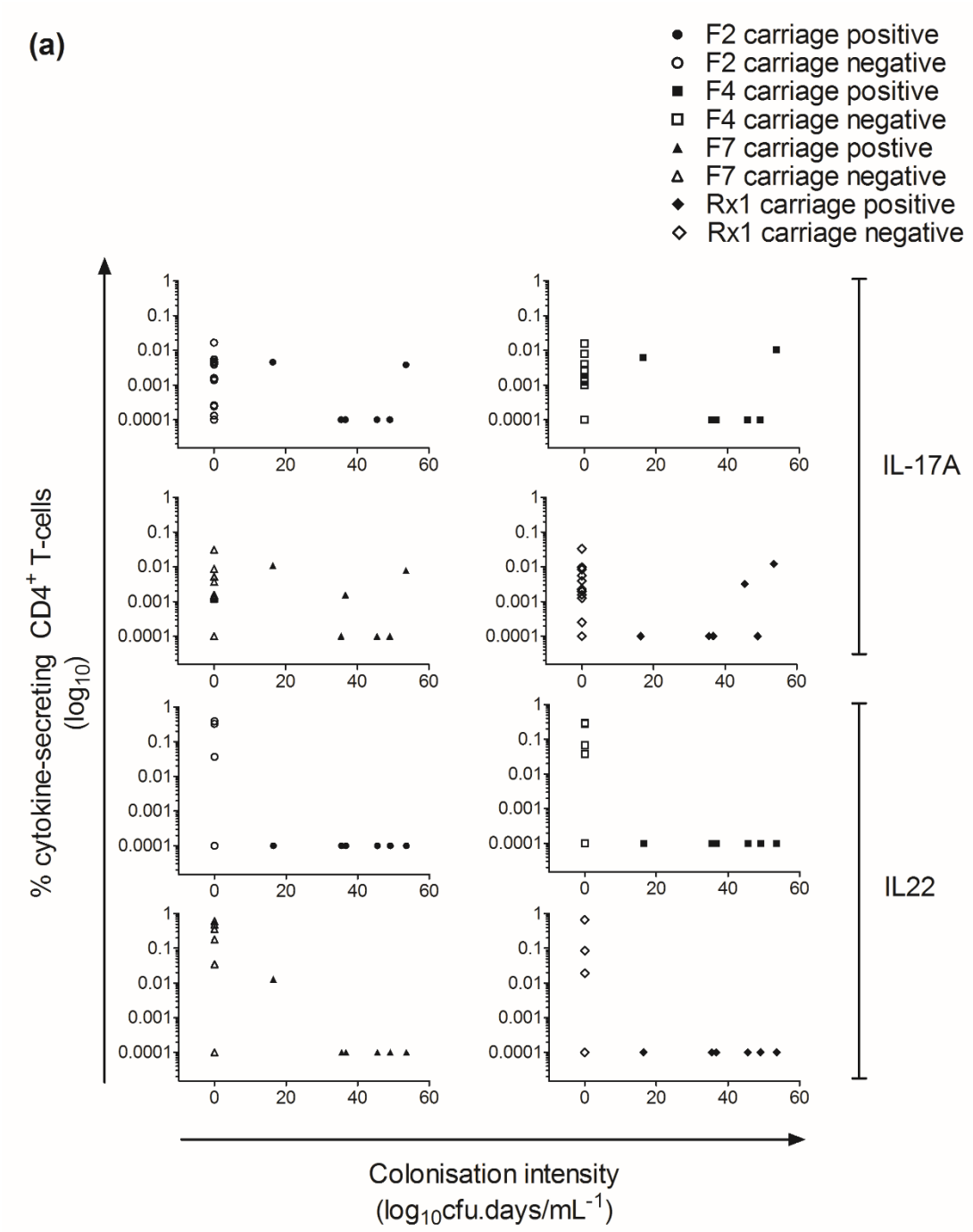
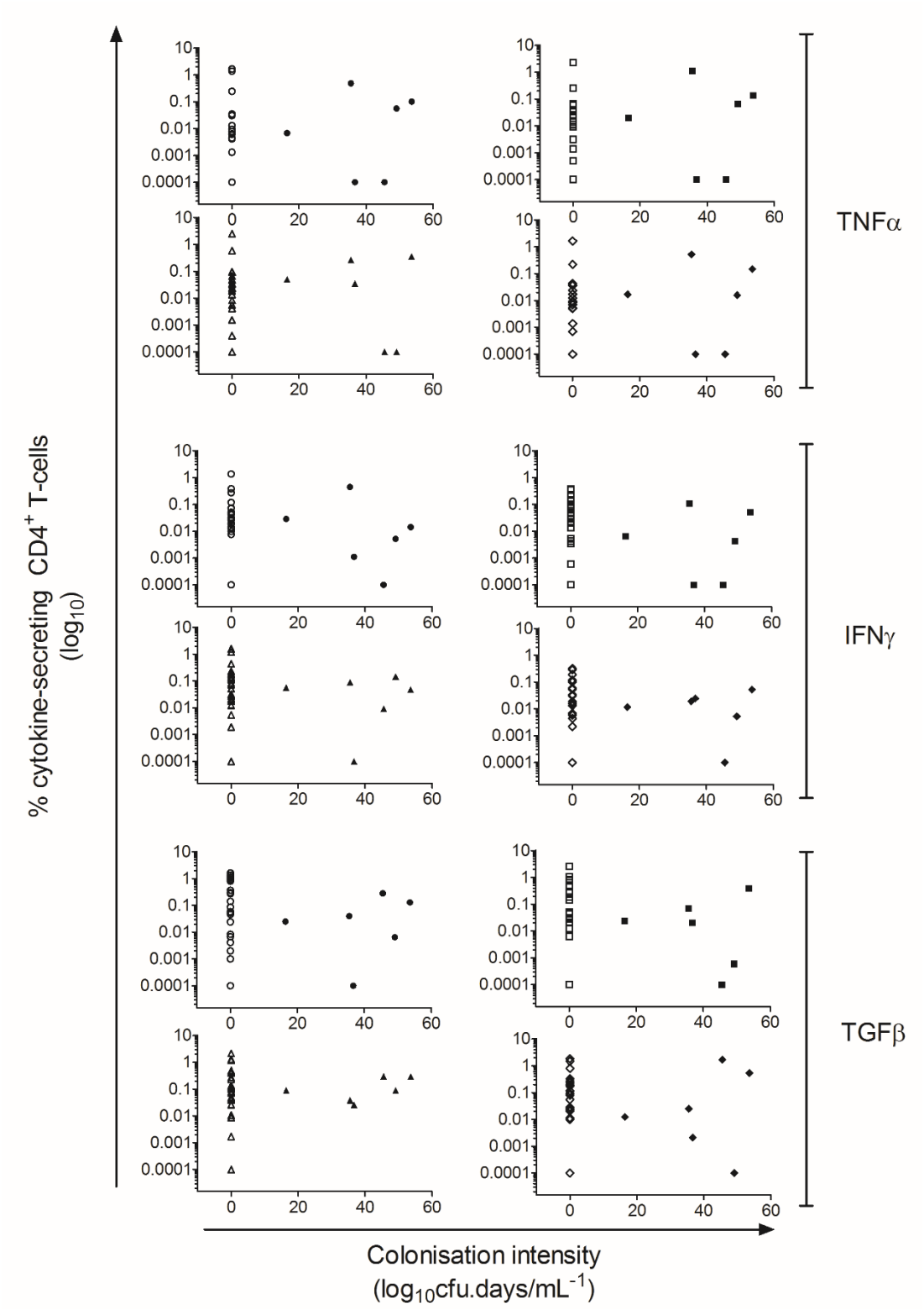


Figure 4.3 Colonisation intensity correlation with PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* (a) by PspA stimulus response (full figure legend for Fig 4.3 panels (a) and (b), page 100).


% cytokine-secreting CD4+ T-cells
(log10)
TNFα
IFNγ
TGFβ
Colonisation intensity
(log10cfu.days/mL-1)

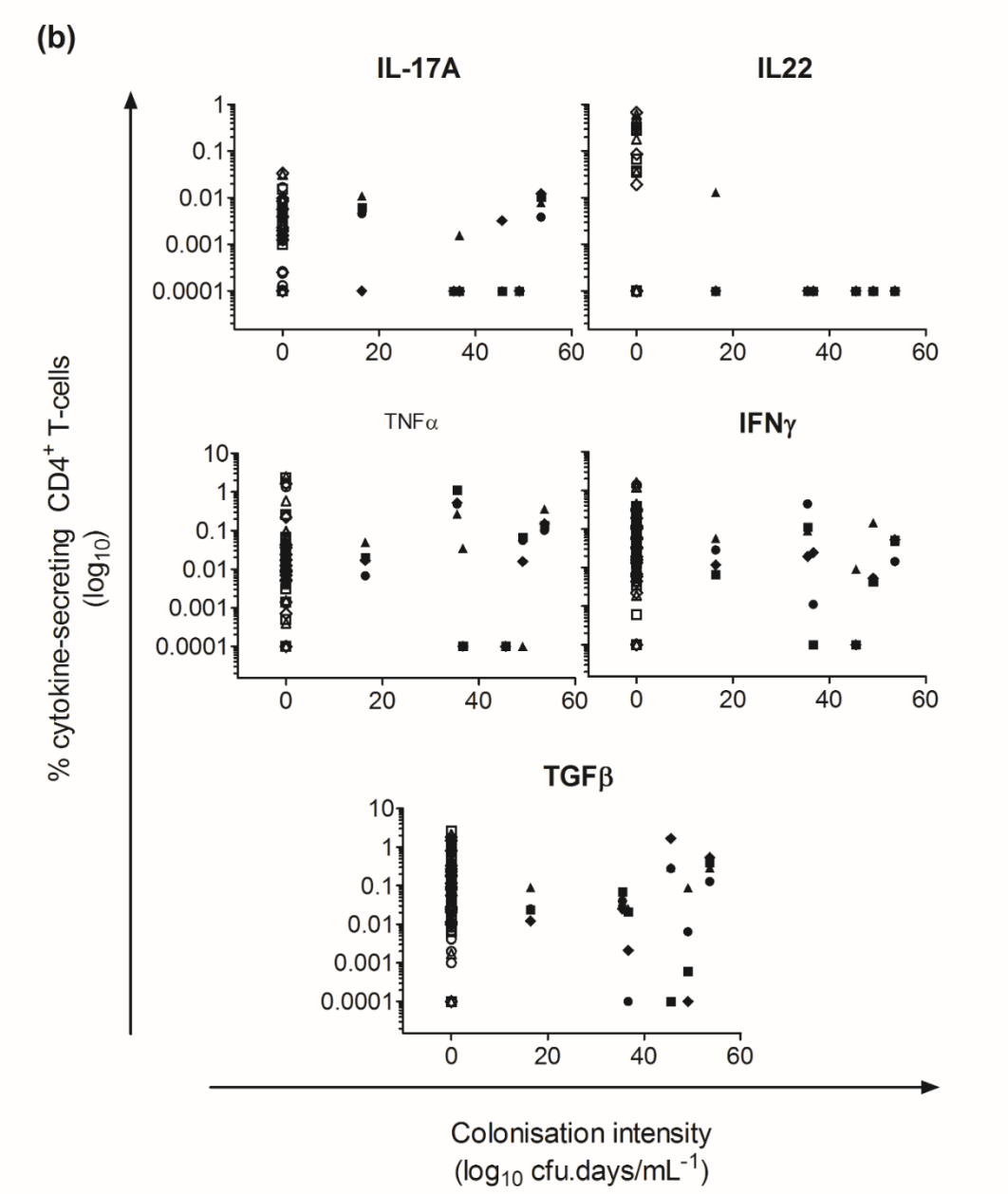


Figure 4.4 Colonisation intensity correlation with PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* (a) by PspA stimulus response (b) total PspA response. Microbiology data from carriage positive volunteers (n = 6) used to calculate intensity of colonisation episode (area under the curve analysis of density of pneumococcus recovered in nasal wash (log_{10}) and duration of colonisation in days), and a correlation analysis conducted against PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* post-inoculation (pilot dataset baseline-corrected combined total volunteer responses - IL17A, IL22, IFNγ, TGFβ - are in bold). Carriage negative volunteer data (n = 32) plotted alongside carriage positive data for descriptive analyses. Stimulation with PspA Fragments 2 (circles), 4 (squares), 7 (triangles) and parent protein, PspA/Rx1 (diamonds). Carriage negative volunteers (open symbols), carriage positive volunteers (closed symbols). Pearson's correlation test performed on log transformed data: % CD4⁺ TGFβ-colonisation intensity correlation alone, $p < 0.05$.

We plotted both carriage negative (open symbols) and carriage positive (closed symbols) volunteer PspA-specific cytokine-secreting $CD4^+$ T-cell responses to enable descriptive exploratory analysis of the relationship between carriage and immune response to *S. pneumoniae* (Figure 4.4). Correlation analysis tests were performed on colonisation intensity and carriage positive volunteer $CD4^+$ T-cell responses to *S. pneumoniae* post-inoculation (baseline corrected). Fragment-specific responses were plotted (Figure 4.4a), but overlapped. Therefore, analysis was performed on combined all stimulus $CD4^+$ T-cell responses to *S. pneumoniae* (Figure 4.4b).

We did not observe a correlation between combined antigen-specific $CD4^+$ Th17-associated (cytokines IL-17A and IL22), inflammatory (TNFα), and Th1-associated (IFNγ) responses to *S. pneum*oniae and colonisation intensity post-inoculation ($p > 0.05$) (Figure 4.4). However, we observed a significant weak negative correlation between post-inoculation PspA-specific $CD4^+TGF\beta^+$ T-cells (T-regulatory cell-associated) and colonisation intensity ($r = -0.23$, $p = 0.04$). We note the limited number of carriage positive volunteer data points with which we conducted correlation analyses ($n = 6$).

Therefore, antigen-specific $CD4^+$ T-regulatory cell-associated responses to *S. pneumoniae* correlated with colonisation intensity: we observed a weak negative correlation between $CD4^+TGF\beta^+$ T-cell responses and post-inoculation carriage intensity, reiterating a role in regulation of colonisation in healthy adults.

4.4 Discussion

We have demonstrated the ability to detect PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* in the blood of healthy adults pre-and post-inoculation. Post-inoculation Fragment 2 and 7-specific $CD4^+TNF\alpha^+$ responses were boosted from pre-existing levels in carriage positive volunteers alone, highlighting these responses as markers of pneumococcal carriage and therefore disease susceptibility, playing a role in response to carriage. PspA-specific $CD4^+TGF\beta^+$ responses to *S. pneumoniae* displayed a weakly negative correlation to post-inoculation carriage intensity (compound measure of density and duration), reiterating a role in regulation of colonisation in healthy adults.

4.4.1 Questions answered: study in field context

Moffitt and colleagues (2011) demonstrated antigen-specific Th17 immunity as a factor in prevention of pneumococcal colonisation in mice. Similarly, Gray *et al*. (2014) implicated an elevated pneumococcal antigen (pneumolysin domain 4)-mediated Th17 immunity in protection from pneumococcal presence in nasal-associated lymphoid tissue of children. In this study, we did not assess a role of pre-existing pneumococcal antigen-specific $CD4^+$ T-cell responses in protection from carriage acquisition in healthy adults due to this study being carried out within a PCV vaccine trial (Collins *et al*., 2015). Although PCV vaccination does not impact PspA immunity (Ditse *et al*., 2013) the predominant vaccine-induced protective anti-polysaccharide IgG precluded our ability to investigate potential baseline protective PspA-specific $CD4^+$ T-cell immune responses to *S. pneumoniae*.

A similar finding to ours however, was demonstrated in a murine study: Cohen *et al*., (2011) showed that pneumococcal exposure primed for an inflammatory Th17 response during challenge, but this was not required for the prevention of invasive disease. This finding is in line with what Wright and colleagues (2013) and we (Section 3.3) have previously described: although nasal inoculation with *S. pneumoniae* boosts immunity as demonstrated by increased cross-reactive, cross-functional IgG to PspA, this does not play a role in protection against heterologous strain carriage re-acquisition. Boosted PspA-specific immunity to *S. pneumoniae* therefore could serve as a marker of pneumococcal exposure.

The carriage positive volunteer boost in post-inoculation F2 and F7-specific inflammatory $CD4^+TNF\alpha^+$ immune response to *S. pneumoniae* points towards a role in response to carriage, and highlights boosted PspA fragment-specific $CD4^+TNF\alpha^+$ responses as potential markers of prolonged pneumococcal exposure and therefore infection susceptibility. These data align with our previous observations of a reduced Fragment 2 and 7-specific IgG in older adults compared to younger adults (Owugha *et al*., in preparation), where older populations are known to have a lower rates of carriage (Almeida *et al*., 2014).

The parent regions of PspA strain Rx1 fragments 2 and 7 have been previously reported as playing a role in pneumococcal immunity. Fragment 2 is located within the alpha-helical PspA region, a highly studied domain due to early identification that immunisation with epitopes in this region protect against fatal murine pneumococcal infection (McDaniel *et al*., 1994). Further work confirming the protective properties of immunity to the alpha helical regions of PspA include: observations of genetic immunisation encoding the alpha helical region protecting mice against lethal challenge (Bosarge *et al*., 2001), and observations of murine infection protection on immunisation (Roche *et al*., 2003).

Fragment 7 originates from the proline-rich region of PspA. The ability of F7-including non-proline block binding monoclonal antibody MAb 1A4.7, to protect mice against morbidity from pneumococcal infection has been reported (Daniels *et al*., 2010). Additionally, in children colonised by *S. pneumoniae*, the generation of proline-rich region-specific antibodies has been observed (Melin *et al*., 2012).

The importance of Th17 and Th1 immunity in *S. pneumoniae* infection (marked by cytokines IL-17A and IFNγ respectively) has been previously demonstrated. Examples include observations of an inverse correlation between IL-17A levels and pneumococcal density in Fijian children (Hoe *et al*., 2015), and up-regulation of IFNγ-related defence genes which associated with protection from pneumococcal pneumonia in mice (Marques *et al*., 2012). We did not identify PspA as a generator of robust Th1 and Th17-associated responses with a role in reduction of pneumococcal carriage density and duration in healthy adults. It is important to note here that the negative correlation observed between pneumococcal density in humans and IL-17A levels by Hoe and co-workers (2015) was not identified as originating from $CD4^+$

T-cells: the cellular source may have been from other T-cell subsets e.g. $CD8^+$ and γδ T-cells, or a range of innate immune populations including neutrophils (Cua and Tato, 2010).

We propose that PspA-specific $CD4^+TGF\beta^+$ responses may play a role in regulating or controlling carriage intensity, furthering pneumococcal-specific observations of Treg-mediated control of and association with, pneumococcal density in upper respiratory tissue of humans and mice (Neill *et al*., 2014, Zhang *et al*., 2011). Our demonstration of PspA-specific $CD4^+TNF\alpha^+$ response as a marker of carriage aligns with previous findings of TNF playing a role in regulation of infection severity in mice (Kirby *et al*., 2005).

4.4.2 Questions raised: study limitations

The nature of investigating immune responses to *S. pneumoniae* in the human host compared to murine models is complex, as no individual is naïve: previous pneumococcal exposure is difficult to account for, and therefore factor into immunological analyses. Additionally, sampling circulating tissue and not that of the site of colonisation may not be optimal to explore protective responses against nasal pneumococcal carriage. However, the advantage of a controlled, measurable and equal pneumococcal inoculation dose is a distinct advantage of the EHPC model, and current investigations are focussed on sampling at the site of inoculation, proximal to that of colonisation at the nasal mucosal tissue (Jochems *et al*., 2017).

Here we demonstrate for the first time, a correlation between pneumococcal antigen-specific $CD4^+TGF\beta^+$ T-cell responses to *S. pneumoniae*, and regulation or control of experimental pneumococcal colonisation in humans. A notable limitation however, was the number of pneumococcal carriers available to conduct the analysis (n = 6), and therefore limited range of colonisation intensities. This study was carried out within a PCV vaccine trial with high efficacy against pneumococcal colonisation, hence the low availability of carriage positive volunteers. An increase in sample size and range of inoculation doses would allow for a larger distribution of carriage positive volunteer density measurements, and therefore more confident conclusions of immune regulators of colonisation intensity.

4.4.3 Questions posed: further work and study implications

The ability to detect protein-specific $CD4^+$ T-cell responses to *S. pneumoniae* and dissect biological relevance relating protection against pneumococcal disease via its precursor nasopharyngeal carriage (using the EHPC model), is key for the testing of novel, broadly-protective vaccine candidates in the natural host. Various pneumococcal vaccines under development take a multiple protein target approach to minimise and slow pneumococcal vaccine escape (Kothari *et al*., 2014, Uraki *et al*., 2015): the ability to measure whether PspA constructs stimulate $CD4^+$ T-cell responses to *S. pneumoniae* is of importance for potential inclusion in these strategies. Additionally, the ability of PspA fragments to induce a greater measurable $CD4^+$ T-cell responses compared to the whole molecule, not only avoids potential structural barriers of similarities to human cardiac myosin, but provides examples of PspA peptide constructs to be included in multiple pneumococcal protein vaccination strategies which look to include T-cell immunogens.

Pneumococcal carriage in healthy adults is immunising and results in increased IgG levels to several protein antigens including PspA (Ferreira, 2013, Wright *et al*., 2013, Ferreira *et al*., 2013). Pneumococcal inoculation alone without carriage induces a pneumococcal-specific Th17-associated cellular immune response (Wright *et al*., 2012). Here we demonstrated that pneumococcal carriage increased PspA-specific $CD4^+TNF\alpha^+$ recall responses to *S. pneumoniae*, therefore played a component role in $CD4^+$ T-cell-mediated inflammatory response to carriage.

Pneumococcal protein-specific $CD4^+$ T-cell responses to *S. pneumoniae* in peripheral blood of humans is of utility as *S. pneumoniae* causes blood-borne diseases. However, the tissue-specific nature of pneumococcal $CD4^+$ T-cell immunity (Jambo *et al*., 2011), highlights the need to investigate $CD4^+$ T-cell responses at relevant sites. With respect to vaccines against pneumonia, this implicates measuring protein-specific $CD4^+$ T-cell responses in the lung.

CHAPTER 5

BAL PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae*

5 BAL PspA-specific CD4$^+$ T-cell responses to *S. pneumoniae*

5.1 Introduction

Streptococcus pneumoniae is the causative organism of a range of mucosal syndromes and diseases including otitis media, sinusitis and pneumonia. The existing 23-valent pneumococcal polysaccharide vaccine (23-PPV) recommended for use in adult groups and the elderly, does not protect against pneumonia (Bonten *et al*., 2015). The conjugate vaccines protect well against invasive pneumococcal disease (IPD) in children, but less well against mucosal diseases including pneumonia (Cutts *et al*., 2005) and otitis media (Rodrigues *et al*., 2013b).

The lung is a specialised immune-regulated environment with a large mucosal surface. Pneumococcal-specific immune responses have been demonstrated as important in defence against *S. pneumoniae* at several mucosal sites including the upper respiratory tract/nasal associated lymphoid tissue (Pido-Lopez *et al*., 2011, Zhang *et al*., 2011). Although we know pneumococcal-specific CD4$^+$ T-cell responses are generated in the human lung (Wright *et al*., 2013), little is known about protein-specific CD4$^+$ T-cell immunity to the pneumococcus at this mucosal site in humans.

Understanding pulmonary immune memory is important as pneumococcal-specific CD4$^+$ T-cell responses have been shown to be compartmentalised (Jambo *et al*., 2011), and vaccine efficacy may be dependent on tissue-specific effector memory. Furthermore, an understanding of beneficial immune responses to *S. pneumoniae* will aid in identification of human populations in which these are lacking, and consequently suffer from critical exacerbations, such as chronic lung disease patients (Chodosh, 1987). Targeting vaccination towards these at risk groups may further reduce the morbidity and mortality associated with pneumococcal lung disease. Therefore, the ability to detect antigen-specific CD4$^+$ T-cell responses in the lung within the EHPC model, is pertinent in the testing of novel vaccine candidates at this important and relevant mucosal site.

Here we investigate PspA-specific $CD4^+$ T-cell immune responses to *S. pneumoniae* in the adult lung, and associate them with recent pneumococcal nasopharyngeal carriage status in healthy adults exposed to *S. pneumoniae* in the EHPC model.

Specifically, our research questions were:

1. Do non-PspA-specific pulmonary $CD4^+$ T-cell cytokine levels associate with carriage status?
2. Are PspA-specific immune responses to *S. pneumoniae* detectable in pulmonary $CD4^+$ T-cells?
3. Does carriage augment PspA-specific responses to *S. pneumoniae* in pulmonary $CD4^+$ T-cells?
4. Do PspA-specific responses to *S. pneumoniae* in pulmonary $CD4^+$ T-cells correlate with carriage intensity?
5. Are PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* of different amplitude in the lung and blood?

5.2 Materials and methods

5.2.1 BAL tissue sample collection, processing and *ex vivo* stimulation

Bronchoalveolar lavage (BAL) samples were collected as previously described (Collins *et al*., 2014). The non-adherent cell fraction containing lymphocytes, was isolated and stimulated as previously described (Section 2.4.2). Briefly, BAL samples were obtained an average of 67 days post-inoculation by bronchoscopy, filtered to remove mucus and whole BAL separated by centrifugation at 470 x *g* for 10 minutes. The resultant cellular pellet was re-suspended, seeded and incubated at 37°C, 5% CO_2 for an average of 3 hours to promote macrophage adhesion.

Non-adherent cells were collected, washed and plated at a count of 200 – 500,000 cells/mL into a 96-well plate. Cells were left unstimulated (for background subtraction of non-specific cytokine secretion), and separately stimulated for 16 hours with 8 µg/mL PspA antigens

(PspA/Rx1, Fragments 2, 4 and 7). After 2 hours of incubation, protein secretion was inhibited as previously described (Section 2.4.2.2), for intracellular cytokine secretion analysis by Flow cytometry (Section 5.2.3).

5.2.2 Blood sample collection, processing and *ex vivo* stimulation

Blood samples were collected at a matched time point to BAL. Peripheral blood mononuclear cells were isolated and stimulated as previously described (Section 2.4.2). Briefly, PBMCs were isolated by density-mediated centrifugation, washed twice and re-suspended in complete media. Cells were seeded for stimulation (with 4 µg/mL pneumococcal antigens PspA/Rx1, Fragments 2, 4 and 7, and 0.5 µg/mL positive control Staphylococcus Enterotoxin B) at a concentration of 1 x 10^6 PBMCs/mL, in addition to a negative unstimulated assay control (for background subtraction). Cytokine secretion was prevented by addition of monensin and brefeldin.

5.2.3 Flow Cytometry

Cells were harvested, stained and analysed for intracellular cytokine production as previously described (Section 2.4.3) Briefly, cells were harvested by centrifugation, stained for surface phenotyping and intracellular cytokine production, and acquired on a BD LSR II flow cytometer. Viable $CD3^+CD4^+CD69^+$ T-cells (hereafter described as $CD4^+$ T-cells) and IL-17A, IL-22, TNFα, IFNγ and TGFβ secretors, were identified using FlowJo software v10 (Treestar, Oregon, USA). Pestle v1.7 and SPICE v.5.2 software (Mario Roederer, Vaccine Research Centre, NIAID, NIH) were employed for background subtraction and data mining respectively. The gating strategy is presented in Figure 2.3.

5.2.4 Statistical Analysis

Data analysis was conducted as previously described (Section 2.4.4). Briefly, stimulus-specific cytokine secreting $CD4^+$ T-cell data underwent initial analysis by One-way ANOVA with

Dunnett's post-hoc testing, and calculation of the average geometric mean fold change (FC) per volunteer across each stimulus (average fold change all stimuli).

Subsequent exploratory analysis employed log-transformed percentage cytokine-secreting $CD4^+$ T-cell data which followed a Gaussian distribution, therefore parametric tests were employed, namely t-tests both paired and unpaired, and Pearson's correlation tests. Graphical representation and statistical analyses were performed using GraphPad Prism v5.0 (California, USA). Differences were considered significant at $p < 0.05$ (two-tailed).

5.3 Results

5.3.1 Non-PspA-specific pulmonary $CD4^+$ T-lymphocyte cytokine levels and association with carriage status

To investigate whether non-PspA-specific $CD4^+$ T-cell cytokine levels associated with post-inoculation carriage status, we compared unstimulated pulmonary $CD4^+$ T-cell cytokine secretion in carriage negative and positive individuals.

We observed no statistical difference in non-PspA-specific $CD4^+$ T-lymphocyte cytokine-secretion between carriage negative (n = 6) and positive volunteers (n = 8) (Figure 5.1). However, volunteer numbers were few, therefore our ability to detect small differences between groups was low. Hence, we note a reduced level of non-PspA-specific $CD4^+$ $IL\text{-}17A^+$ secretion in the carriage positive cohort compared to the negative (difference between means [95% CI]: 0.49 ± 0.54 [-0.67 to 1.66] $p = 0.37$), and the reverse trend on measurement of non-PspA-specific $CD4^+$ $TGF\beta^+$ secretion (-0.74 ± 0.47 [-1.76 to 0.29] $p = 0.14$). Carriage negative and positive $CD4^+$ T-cell secretion levels were similar for IL22 (0.18 ± 0.30 [-0.54 to 0.90] $p = 0.57$), TNFα (-0.71 ± 0.56 [-1.93 to 0.50] $p = 0.23$) and IFNγ (0.09 ± 0.69 [-1.42 to 1.60] $p = 0.89$).

Therefore, we did not detect significant non-PspA-specific pulmonary $CD4^+$ T-cell cytokine levels associated with carriage status. However, we detected higher carriage positive volunteer $CD4^+$ $TGF\beta^+$ secretion compared to carriage negative volunteers, where $CD4^+$ IL-

17A^{+} secretion was reciprocally lower in carriage positive compared to negative volunteers. This reflected the immune paradigm of an inverse relationship between inflammatory Th17/T-regulatory responses, and may indicate a mechanism of preventing excessive inflammation in the lung of a population with increased risk of pneumococcal aspiration to the lung (carriage positive volunteers).

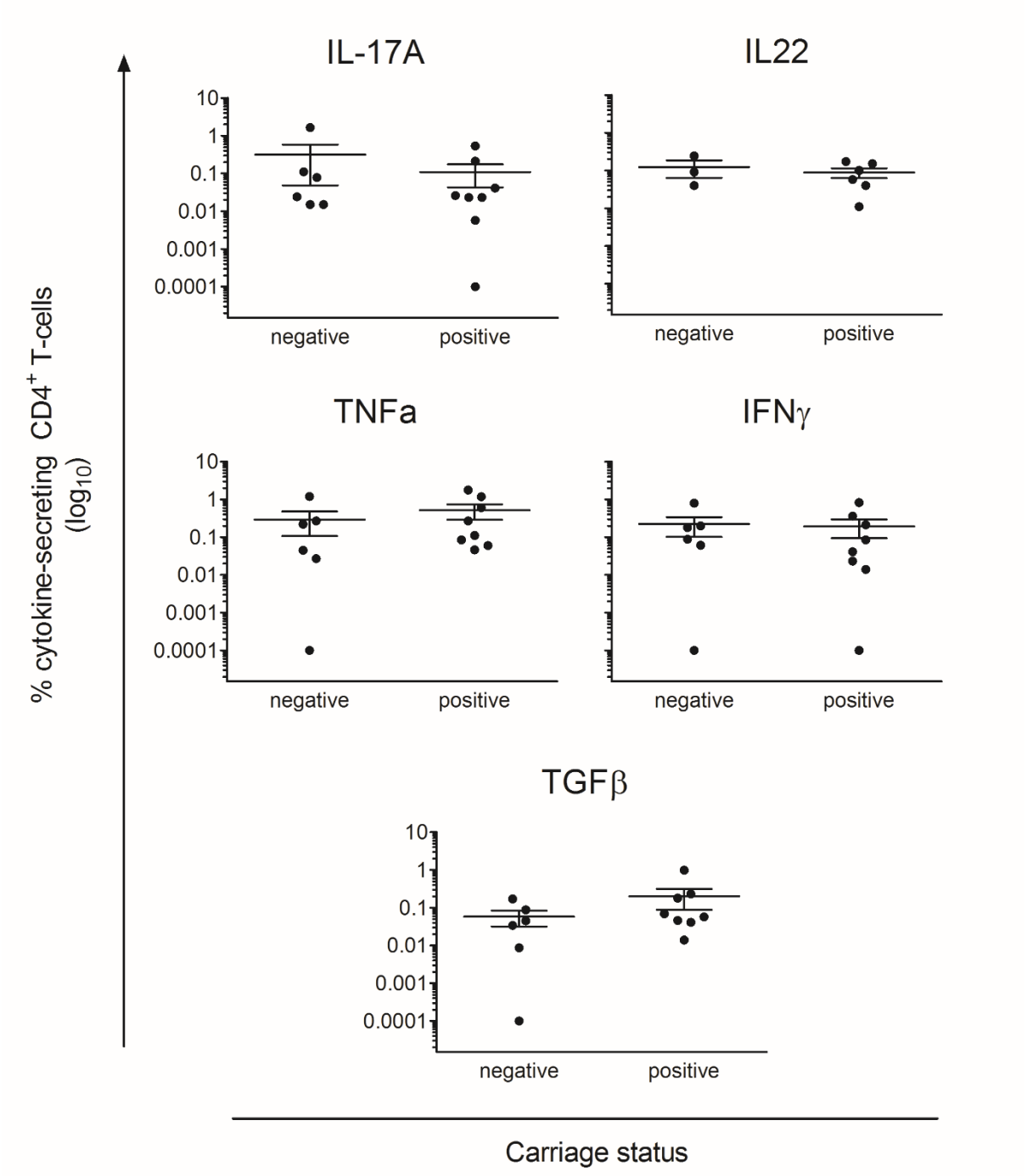


Figure 5.1 Non-PspA-specific CD4⁺ T-cell cytokine levels in the human lung. Unstimulated CD4⁺ T-lymphocyte cytokine secretion in carriage negative (n = 6) and positive (n = 8) volunteers in the lung post *S. pneumoniae* inoculation. Unpaired t-test on log transformed data (all data $p > 0.05$). Horizontal bars represent mean ± SEM.

5.3.2 PspA-specific CD4⁺ T-lymphocyte responses to *S. pneumoniae* in the lung

In order to harness the EHPC model to test novel serotype-independent vaccine candidates at a relevant mucosal site, we assessed the sensitivity of our assay to detect induction of single pneumococcal antigen-specific pulmonary CD4⁺ T-cell responses to *S. pneumoniae*.

Fourteen volunteers underwent bronchoscopy an average of 67 days post-inoculation for obtaining BAL. There was no difference in time elapsed between inoculation and BAL procedure in carriage negative (n = 6, 76.83 ± 27.37 days) and carriage positive volunteer groups (n = 8, 64.63 ± 21.39 days) (p = 0.37, unpaired t-test) (Table 5.1).

Table 5.1 Time elapsed between pneumococcal inoculation and bronchoscopy procedure.

Carriage status	Volunteer number	Time elapsed (days)	Average (days)
Negative	1	31	76.83 ± 27.37
	2	96	
	3	109	
	4	87	
	5	66	
	6	72	
Positive	7	29	64.63 ± 21.39
	8	52	
	9	42	
	10	80	
	11	86	
	12	75	
	13	86	
	14	67	

Average time elapsed measured as mean number of days ± SD.

We detected PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* in BAL: $CD4^+$ $IL\text{-}17A^+$, $IL22^+$, $TNF\alpha^+$ and $IFN\gamma^+$ T-cell proportions had an average fold change (all stimuli) > 1 (Table 5.2). The highest differences in cytokine secretion on PspA-stimulation were observed for Th1 cytokine IFNγ (FC = 2.38) and inflammatory cytokine TNFα (FC = 1.88) whereas no overall difference was observed on PspA-specific $CD4^+TGF\beta^+$-secretion (FC = 0.99).

Table 5.2 Fold change in pulmonary post-inoculation unstimulated to stimulated PspA-specific $CD4^+$ T-cells.

	Average percentage cytokine secreting pulmonary $CD4^+$ T-cells					
	US	F2	F4	F7	PspA/Rx1	Average Fold Change (all stimuli)
IL-17A	0.04 [0.01 to 0.24]	0.12 [0.05 to 0.31]	0.03 [0.00 to 0.20]	0.03 [0.00 to 0.30]	0.02 [0.00 to 0.16]	**1.10** [0.51 to 2.35]
IL22	0.09 [0.05 to 0.16]	0.11 [0.05 to 0.26]	0.09 [0.04 to 0.20]	0.03 [0.00 to 0.32]	0.15 [0.05 to 0.49]	**1.14** [0.68 to 1.91]
TNFα	0.12 [0.02 to 0.75]	0.35 [0.16 to 0.79]	0.22 [0.05 to 1.09]	0.09 [0.01 to 0.85]	0.22 [0.05 to 0.94]	**1.88** [1.25 to 2.83]
IFNγ	0.08 [0.01 to 0.43]	0.24 [0.11 to 0.54]	0.11 [0.03 to 0.49]	0.13 [0.03 to 0.58]	0.11 [0.03 to 0.43]	**2.38** [1.38 to 4.11]
TGFβ	0.05 [0.01 to 0.24]	0.07 [0.01 to 0.41]	0.06 [0.01 to 0.25]	0.03 [0.00 to 0.23]	0.02 [0.00 to 0.14]	**0.99** [0.33 to 2.99]

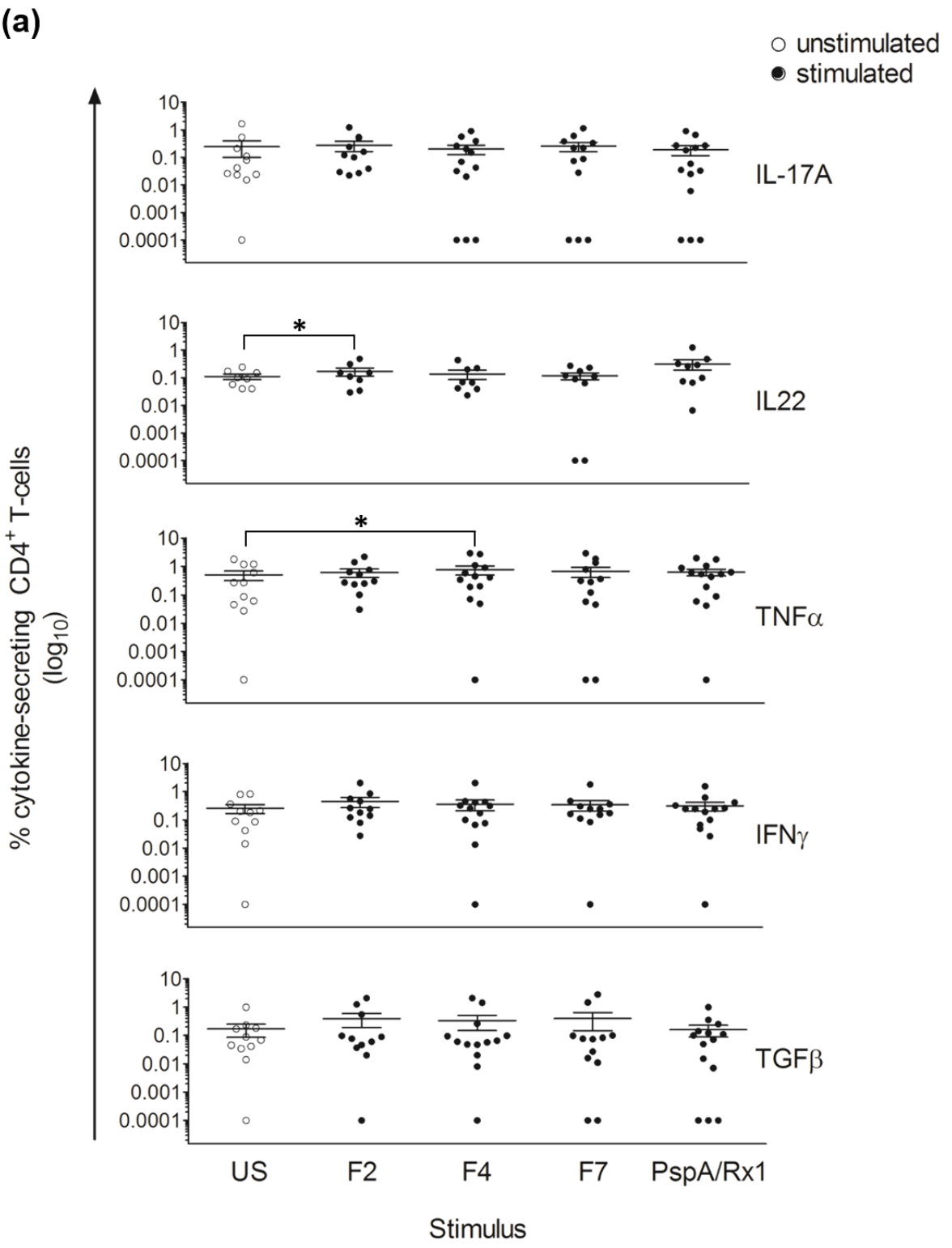
Average percentage cytokine secreting $CD4^+$ T-cells – geometric mean [95% confidence interval of geometric mean]. US – unstimulated; F*n* – fragment *n*. Average fold change all stimuli – mean fold change from unstimulated to stimulated for F2, F4, F7 and PspA/Rx1 stimuli.

However, differences between unstimulated and combined PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* were not statistically different at any one of five cytokines measured: IL-17A, $p = 0.80$; IL22, $p = 0.11$; TNFα, $p = 0.11$; IFNγ, $p = 0.22$; TGFβ, $p = 0.43$ (One-way ANOVA) (Figure 5.2a). On PspA stimulation, a number of volunteer $CD4^+$ T-cell responses fell to undetectable levels: 81% of undetectable cytokine secretion cases were in carriage positive volunteers (n = 13 of 16) (Figure 5.2b).

On employing Dunnett's post-hoc testing to correct for multiple comparisons between the constant unstimulated control measurements and variant antigen-specific conditions, differences in means were detected on F2-specific $CD4^+$ IL22 secretion (mean difference [95%

confidence interval]: -0.36 [-0.69 to -0.03]), and F4-sepcific $CD4^+$ TNFα secretion (-0.29 [-0.56 to -0.02]) (Figure 5.2a). We did not detect a difference between unstimulated and antigen-stimulated cytokine secretion when data were stratified by carriage status ($p > 0.05$) (Figure 5.2b).

Therefore, PspA-specific immune response to *S. pneumoniae* in pulmonary $CD4^+$ T-cells was not statistically different to unstimulated levels. This may reflect the lack of immunodominance of PspA as an antigen in the lung. Consequently, subsequent analyses were exploratory, primarily using data of conditions where $CD4^+$ responses were significant, as a pilot dataset addressing outlined research questions.


(a)
unstimulated
stimulated
% cytokine-secreting CD4+ T-cells (log10)
IL-17A
IL22
TNFα
IFNγ
TGFβ
10
1
0.1
0.01
0.001
0.0001
*
US
F2
F4
F7
PspA/Rx1
Stimulus

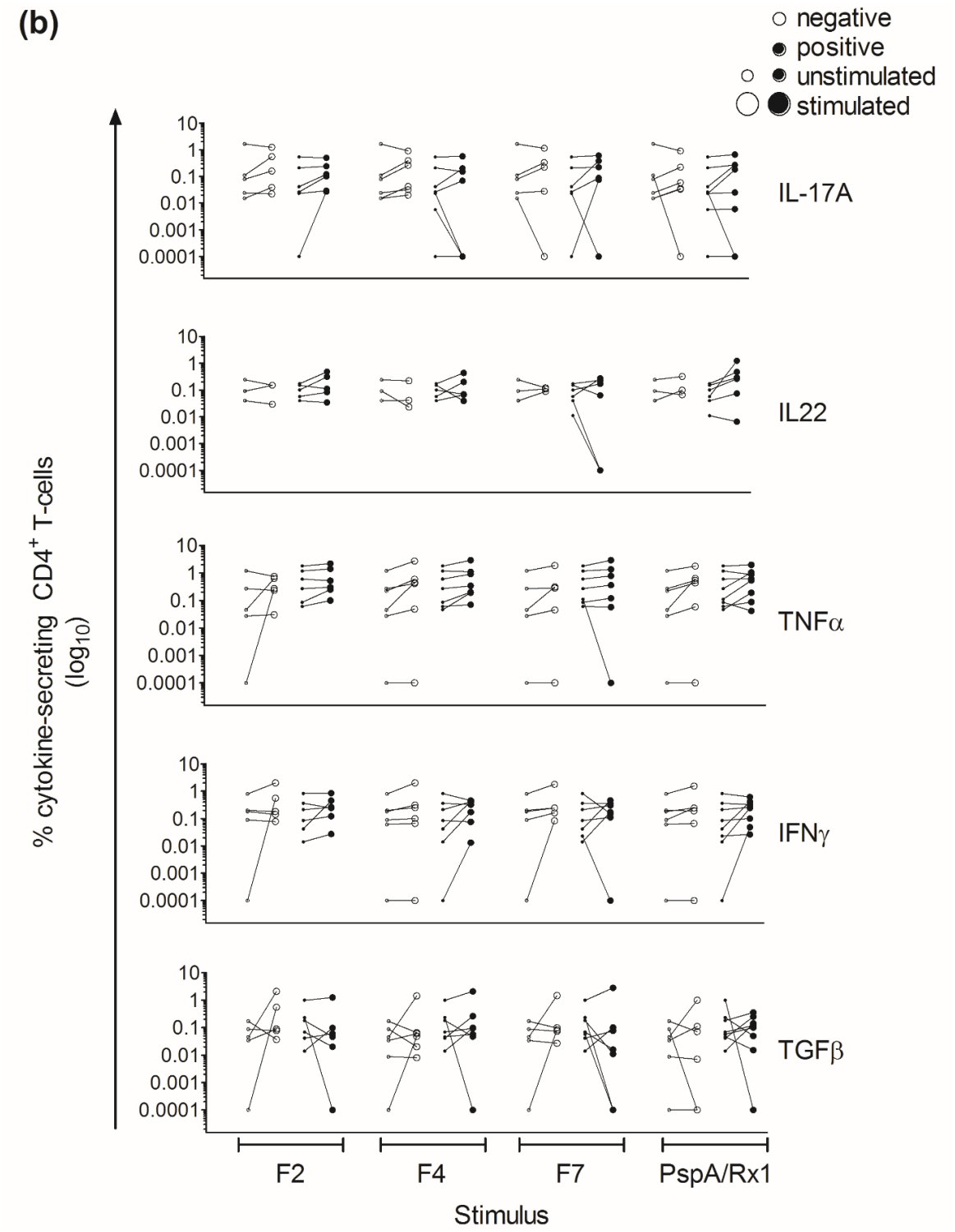


Figure 5.2 PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* in the human lung. CD4⁺ IL-17A, IL22, TNFα, IFNγ and TGFβ secretion in the lung post *S. pneumoniae* inoculation. (a) all volunteers - unstimulated (open circles), PspA-stimulated (closed circles). One-way ANOVA with Dunnett's post-hoc corrections, * = $p < 0.05$ (all ANOVA data $p > 0.05$). (b) by carriage status – carriage negative (open circles), carriage positive (closed circles), unstimulated (small circles), stimulated (large circles). Horizontal bars represent mean ± SEM.

5.3.3 Carriage status and association with PspA-specific pulmonary $CD4^+$ T-cell responses to *S. pneumoniae*

We investigated whether post-inoculation pulmonary PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* differed between carriage negative and positive individuals. We note that these analyses are against a background of inability to detect a significant PspA-specific $CD4^+$ T-cell cytokine response to *S. pneumoniae* in a small dataset (carriage negative: n = 6; carriage positive: n = 8) (Section 5.3.2). Therefore, we present these data as a descriptive analysis to defined research questions. We highlight a pilot dataset where pulmonary PspA-specific $CD4^+$ T-cell cytokine secretion was significant (F2-specific $CD4^+IL22^+$ and F4-specific $CD4^+TNF\alpha^+$ responses) (Figure 5.2).

We observed no association between carriage status and proportion of PspA-specific pulmonary $CD4^+$ T-cells secreting IL-17A, IL22, TNFα, IFNγ or TGFβ ($p > 0.05$, unpaired t-test) (Figure 5.3). Where there was a previously detected significant PspA-specific CD^+ T-cell response to *S. pneumoniae*, we maintained inability to associate carriage status with immune response: percentage F2-specific $CD4^+$ T-cells secreting $IL22^+$ (difference between means [95% CI]: 0.45 ± 1.24 [-2.45 to 3.37] $p = 0.73$), and F4-specific $CD4^+$ T-cells secreting TNFα (0.15 ± 0.78 [-1.58 to 1.87] $p = 0.86$) were not statistically different between carriage negative and positive groups (Figure 5.3).

Therefore, carriage did not augment PspA-specific responses to *S. pneumoniae* in pulmonary $CD4^+$ T-cells. This supports our observation that PspA may not be an immunodominant antigen in the lung.

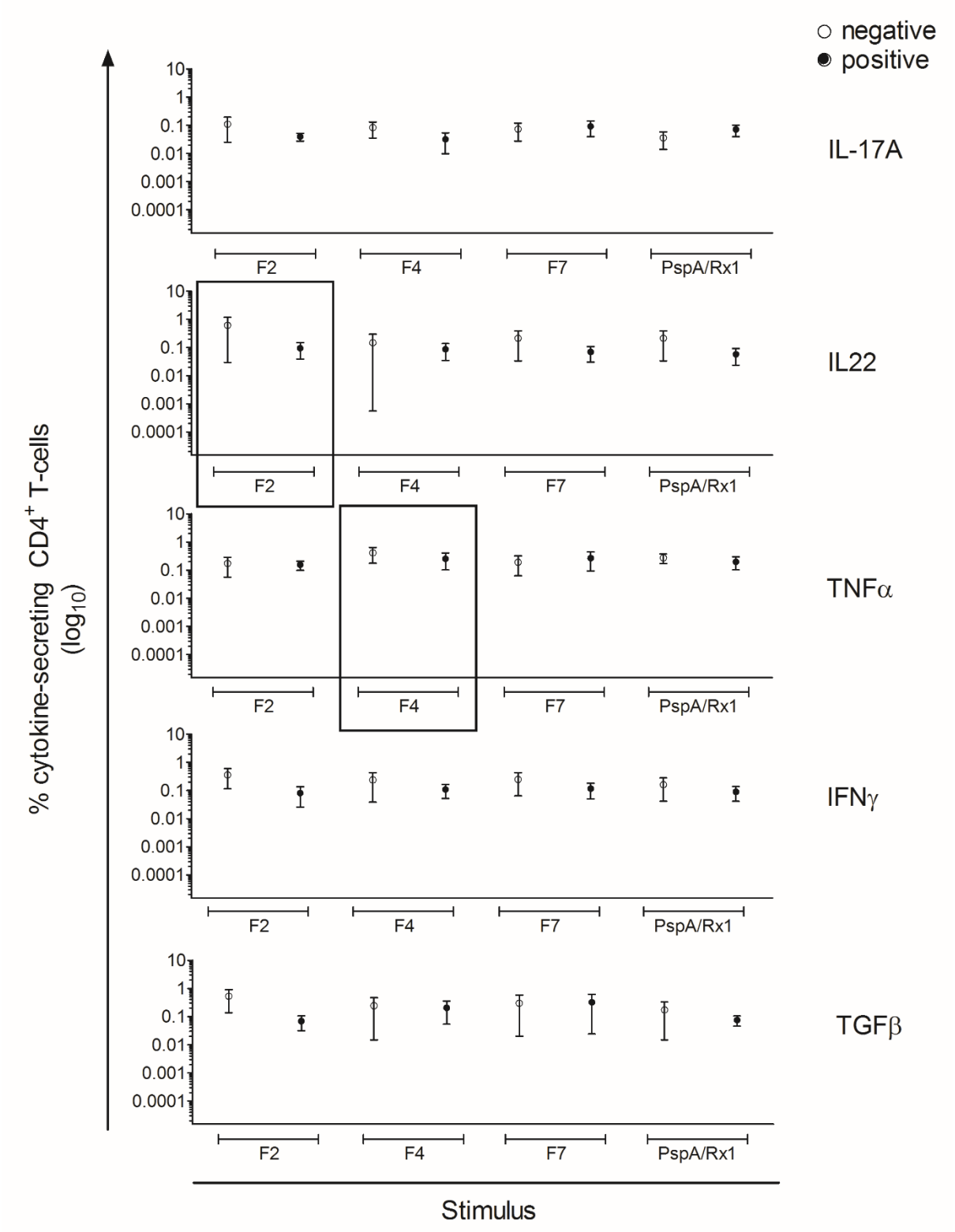


Figure 5.3 Carriage status and PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* in the human lung. Unstimulated-corrected PspA-specific CD4⁺ IL-17A, IL22, TNFα, IFNγ and TGFβ-secretion in the lung of carriage negative (open circles) and positive (closed circles) volunteers post *S. pneumoniae* inoculation. Significant pulmonary PspA-specific CD4⁺ T-cell responses boxed. Unpaired t-test on log-transformed data (all data $p > 0.05$). Mean value plotted, horizontal bars represent ± SEM.

5.3.4 Correlation of pneumococcal colonisation intensity with PspA-specific pulmonary CD4⁺ T-cell responses to *S. pneumoniae*

We calculated a compound, continuous measure of carriage density and duration (area under the curve analysis of density of pneumococcus recovered in nasal wash (log_{10}) and duration of colonisation in days) (Table 5.3) to investigate whether PspA-specific CD4⁺ T-cell cytokine-responses to *S. pneumoniae* correlated with extent of colonisation (colonisation intensity). We note that overall, we did not detect a significant pulmonary PspA-specific CD4⁺ T-cell response to *S. pneumoniae* (unstimulated to stimulated cells, Section 5.3.2), therefore highlight data for statistically significant conditions as a pilot analysis as per defined research questions (F2-specific CD4⁺IL22⁺ and F4-specific CD4⁺TNFα⁺ responses).

Table 5.3 Colonisation intensity from microbiology carriage density and duration data.

	Volunteer							
Duration (days)	1	2	3	4	5	6	7	8
	Density (log_{10}cfu/mL)							
Day 2	5.12	0.65	0.81	2.65	2.15	3.90	3.39	2.35
Day 7	2.86	1.52	0.96	4.71	1.81	2.43	4.51	3.00
Day 17	3.59	3.43	1.07	4.30	-	3.54	2.01	1.46
Day 21	-0.39	4.11	0.33	4.39	3.78	-	-	0.40
	Colonisation intensity (log_{10}cfu.days/mL^{-1})							
	54.00	49.20	16.40	80.30	49.10	36.70	42.50	35.50

Colonisation intensity = Area Under the Curve analysis of density and duration per pneumococcal carriage episode.

We did not detect a correlation between colonisation intensity and F2-specific CD4⁺IL22⁺ T-cell responses to *S. pneumoniae* (r = 0.15, *p* = 0.78) or F4-specific CD4⁺TNFα⁺ responses (r = 0.29, *p* = 0.56) (Figure 5.4). We also did not detect a correlation between IL-17A, IFNγ and TGFβ-secreting PspA-specific CD4⁺ T-cells to *S. pneumoniae* (all stimuli combined) (Figure 5.5), notably against a background of non-statistical difference in detection of differences between unstimulated and stimulated cells.

Therefore, PspA-specific responses to *S. pneumoniae* in pulmonary $CD4^+$ T-cells did not correlate with carriage intensity. This further strengthens observations that PspA may not be an immunodominant antigen in the human adult lung.

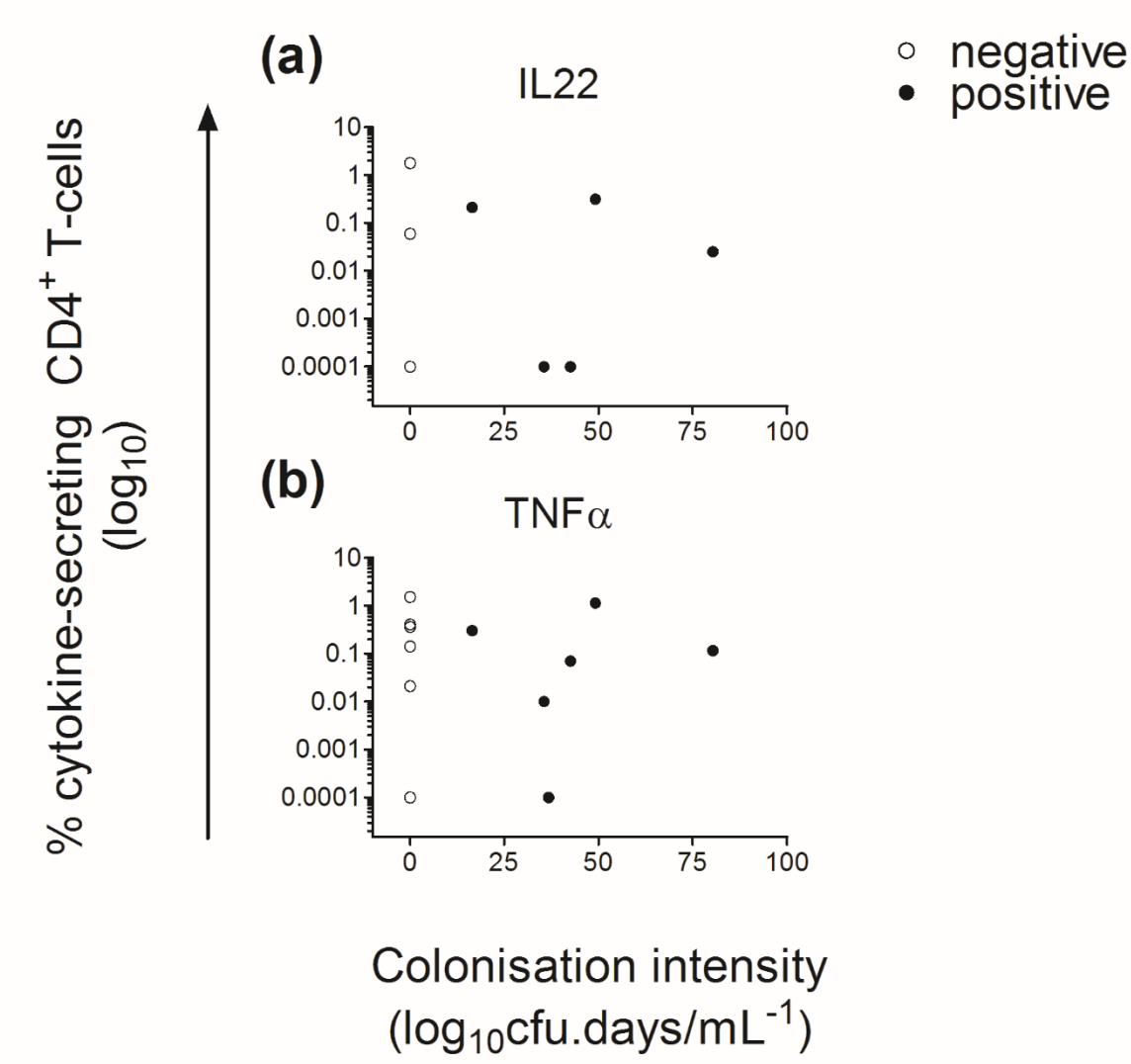


Figure 5.4 Colonisation intensity and PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* in the human lung. Unstimulated-corrected (a) Fragment 2-specific $CD4^+IL22^+$ and (b) Fragment 4-specific $CD4^+TNF\alpha^+$ pulmonary T-cell responses to *S. pneumoniae* post-inoculation. Open symbols - carriage negative volunteers; closed symbols - carriage positive volunteers. Pearson's correlation on log transformed carriage positive volunteer $CD4^+$ T-cell responses (all data $p > 0.05$).

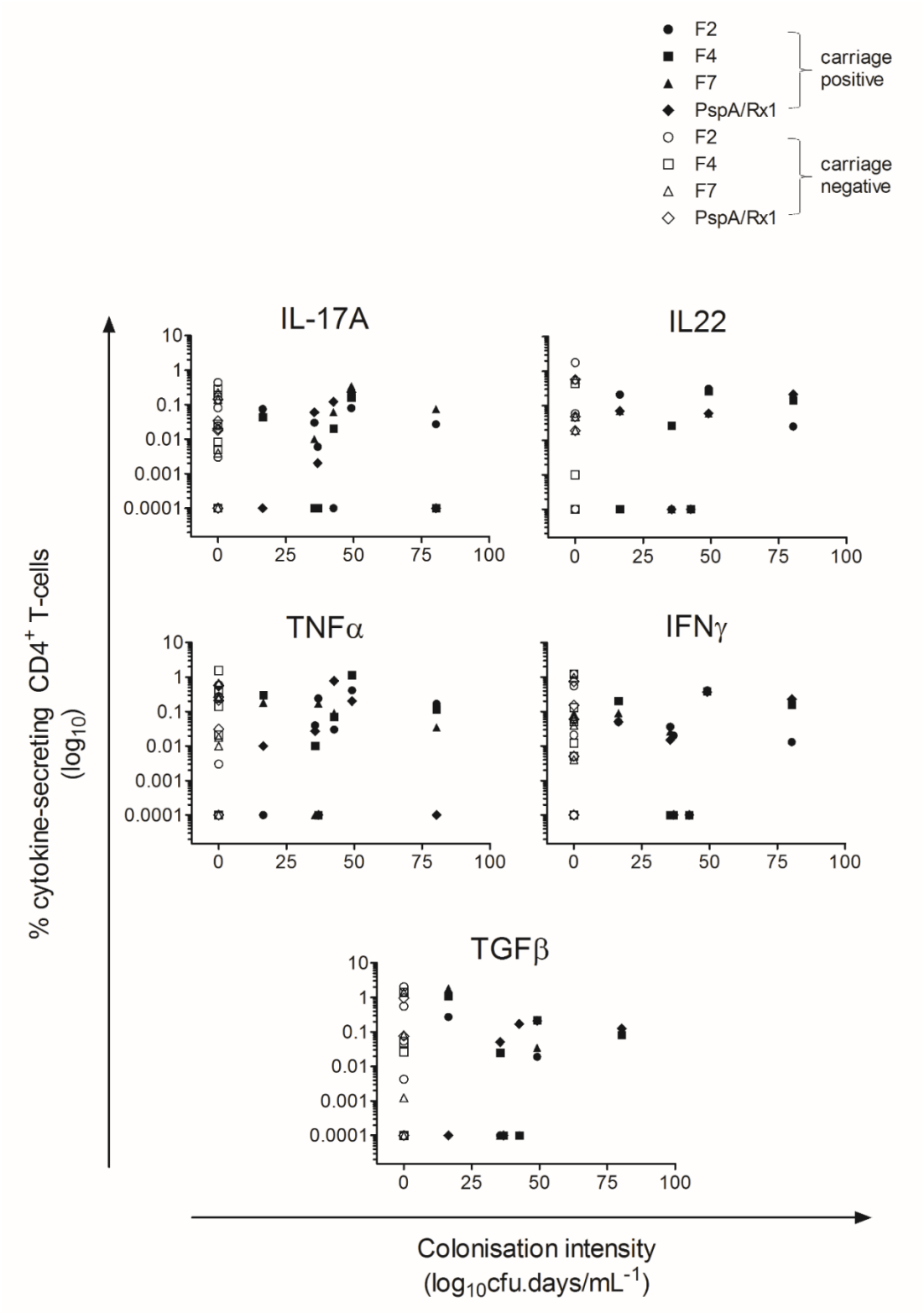


Figure 5.5 Colonisation intensity and PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* in the human lung. PspA-stimulated CD4⁺ IL-17A, IL22, TNFα, IFNγ and TGFβ-secretion in the lung of individuals post *S. pneumoniae* inoculation. Pearson's correlation analysis on log transformed CD4⁺ T-cell data of carriage positive volunteers (all data $p > 0.05$).

5.3.5 PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* in the lung and blood

To investigate whether post-inoculation PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* were of a similar amplitude in the lung and systemically, we compared CD4⁺ T-cell response levels at matching time points in BAL and blood. Where pulmonary responses were significantly detected, we employed data for a pilot analysis of pre-defined questions.

Although pilot dataset F2-specific CD4⁺ IL22 secretion did not significantly differ across BAL and blood tissue (mean of differences [95% CI]: -0.53 [-1.82 to -2.89] $p = 0.56$), F4-specific CD4⁺TNFα secretion was significantly lower in BAL compared to blood (-2.13 [-3.69 to -0.57] $p = 0.01$) (Figure 5.6). Carriage negative volunteer F4-specific CD4⁺TNFα⁺ T-cell responses to *S. pneumoniae* were significantly lower in BAL compared to blood (-2.78 [-4.01 to -1.56] $p < 0.01$) (Figure 5.6).

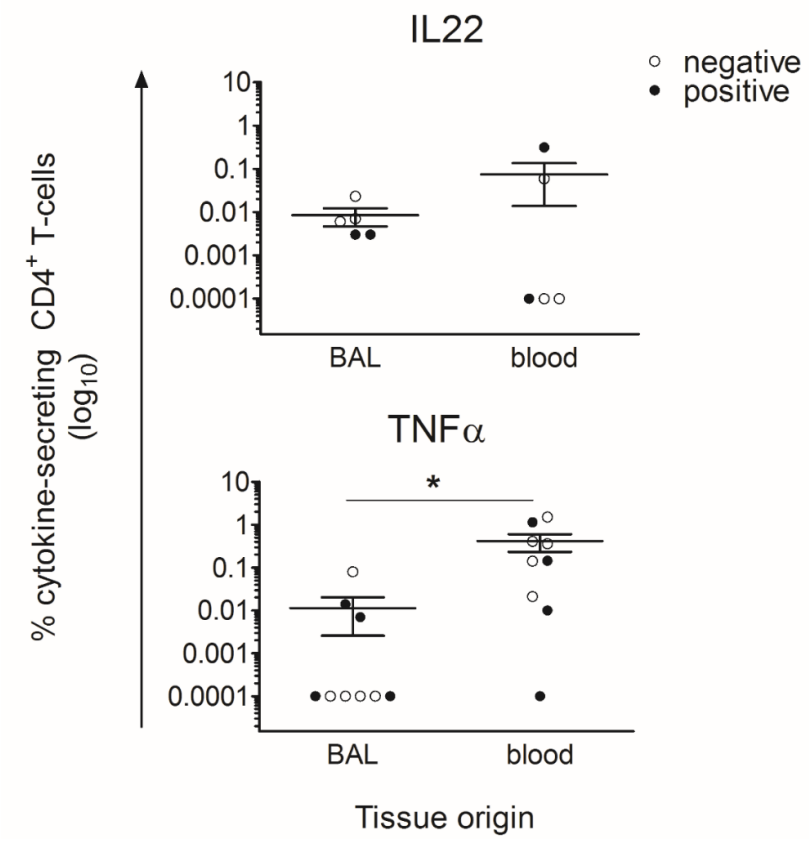


Figure 5.6 Detectable PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* in BAL and blood. Unstimulated-corrected F2-specific CD4⁺IL22⁺ and F4-specific TNFα⁺ T-cell responses to *S. pneumoniae* in the lung and blood of volunteers at matched time-points. Carriage negative (open circles) and carriage positive (closed circles) volunteers. Paired t-test on log transformed data, *$p < 0.05$. Horizontal bars represent mean ± SEM.

We observed a lower combined PspA-specific CD4⁺TNFα secretion in the lung as compared to the blood of volunteers (-1.39 [-2.11 to -0.67] $p < 0.01$) (Figure 5.7): this difference was most marked for carriage positive volunteers (-1.78 [-2.61 to -0.94] $p < 0.01$). We note this result was against a background of non-statistical difference between unstimulated and stimulated blood CD4⁺ T-cells post-inoculation (Figure 5.8), and comparable cytokine detection in matched unstimulated controls (FC = 0.93) (Table 5.4).

Table 5.4 Matched BAL time-point blood fold changes in unstimulated to stimulated PspA-specific CD4⁺ T-cells.

	Average percentage cytokine secreting blood CD4⁺ T-cells					
	US	F2	F4	F7	PspA/Rx1	Average Fold Change (all stimuli)
IL-17A	0.05 [0.01 to 0.21]	0.13 [0.04 to 0.41]	0.24 [0.07 to 0.83]	0.18 [0.05 to 0.68]	0.13 [0.04 to 0.50]	**3.33** [1.75 to 6.32]
IL22	0.04 [0.01 to 0.10]	0.05 [0.02 to 0.11]	0.04 [0.01 to 0.10]	0.05 [0.02 to 0.12]	0.04 [0.01 to 0.13]	**1.11** [0.85 to 1.45]
TNFα	0.03 [0.01 to 0.08]	0.03 [0.01 to 0.09]	0.02 [0.01 to 0.08]	0.03 [0.01 to 0.09]	0.03 [0.01 to 0.11]	**0.93** [0.66 to 1.30]
IFNγ	0.01 [0.01 to 0.03]	0.02 [0.00 to 0.12]	0.04 [0.01 to 0.13]	0.03 [0.01 to 0.10]	0.03 [0.01 to 0.09]	**1.90** [1.37 to 2.64]
TGFβ	0.02 [0.01 to 0.04]	0.01 [0.00 to 0.09]	0.02 [0.00 to 0.11]	0.01 [0.00 to 0.09]	0.02 [0.00 to 0.16]	**0.73** [0.34 to 1.54]

Average percentage cytokine secreting CD4⁺ T-cells – geometric mean [95% confidence interval of geometric mean]. US – unstimulated; F*n* – fragment *n*. Average fold change all stimuli – mean fold change from unstimulated to stimulated for F2, F4, F7 and PspA/Rx1 stimuli.

Similarly, we observed a lower combined PspA-specific Th17-associated immune response in the lung compared to blood: CD4⁺IL-17A secretion was lower in BAL (-0.71 [-1.37 to -0.05] $p = 0.04$) (Figure 5.7): this difference too was most marked for carriage positive volunteers (-0.98 [-2.00 to 0.04] $p = 0.06$). Carriage positive volunteer CD4⁺IL22 secretion was similarly, but not significantly, lower in BAL compared to blood (-0.25 [-1.92 to 1.42] $p = 0.73$). We note these observations are against a background of detectable, but not significant differences in unstimulated to stimulated CD4⁺ T-cells in blood at the same time-point (IL-17A: FC = 3.33, $p = 0.73$; IL22: FC = 1.11, $p = 0.48$) (Table 5.4, Figure 5.8).

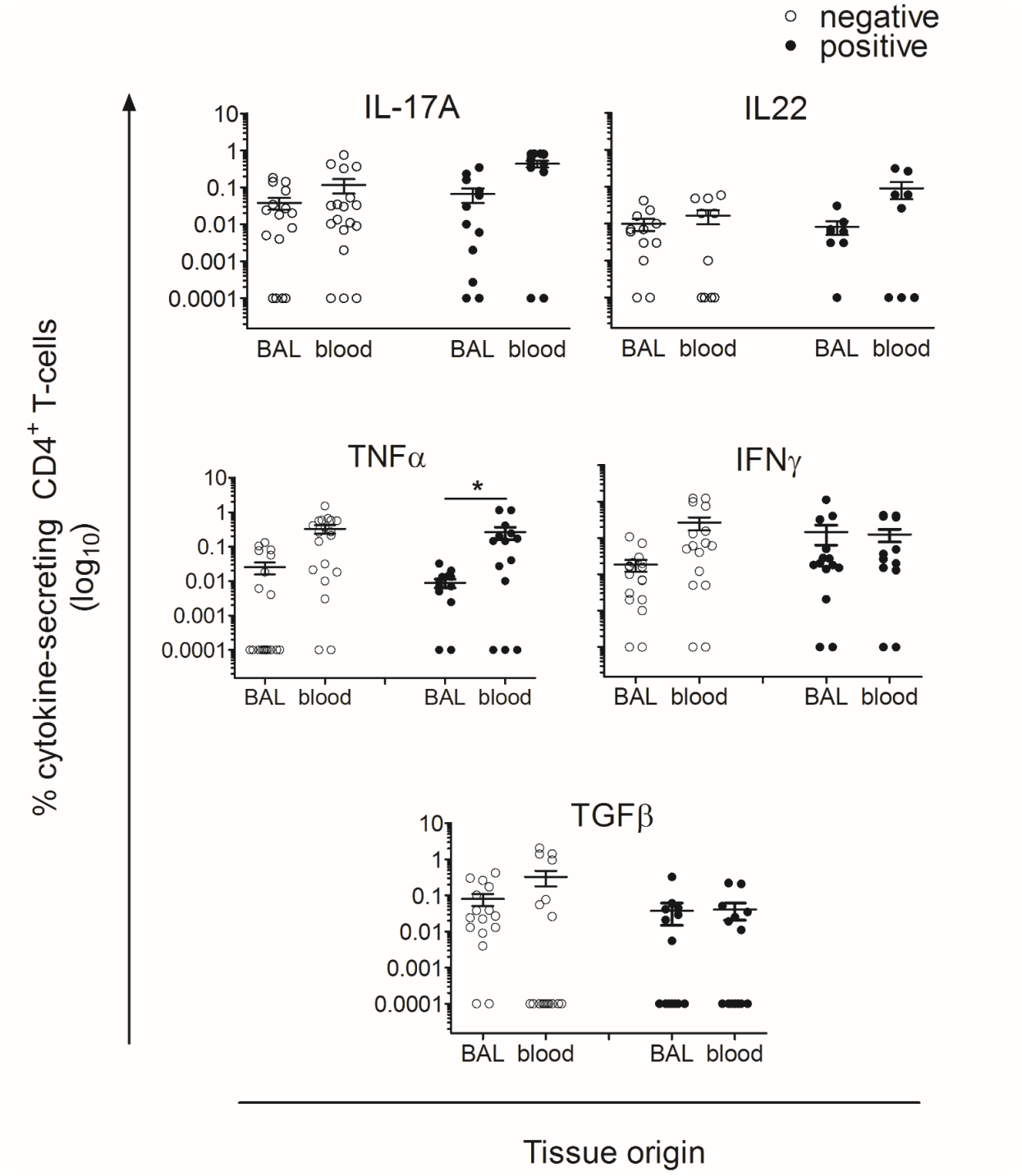
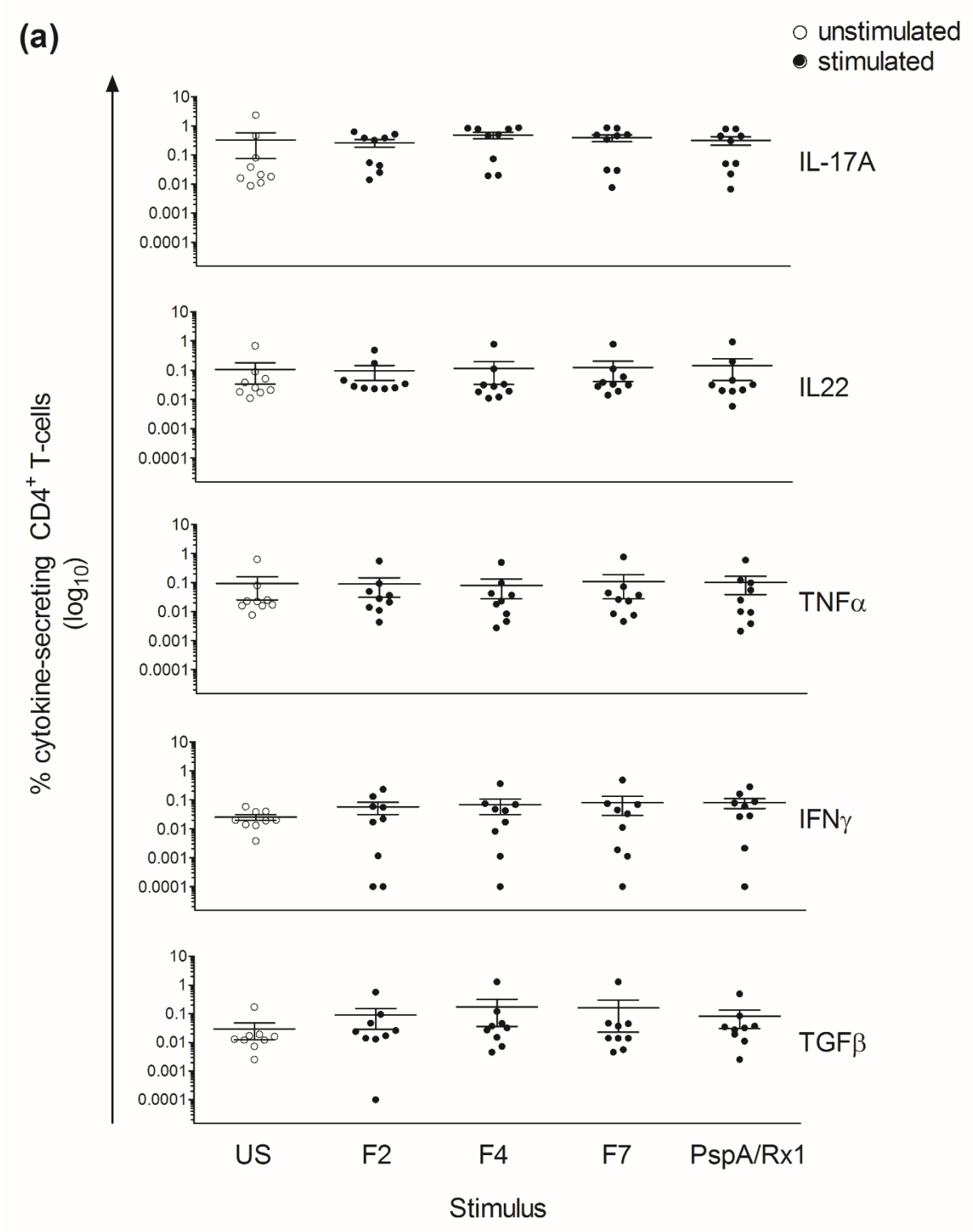


Figure 5.7 PspA-specific CD4⁺ T-cell responses to *S. pneumoniae* in BAL and blood. Unstimulated-corrected PspA-stimulated (all stimuli) CD4⁺ IL-17A, IL22, TNFα, IFNγ and TGFβ-secretion in the lung and blood of volunteers at matched time-points. Paired t-test on log transformed data, *$p < 0.05$. Horizontal bars represent mean ± SEM.

Although the non-statistically significant trend in low BAL, high blood $CD4^+$ T-cell cytokine secretion was evident for carriage negative IFNγ (-0.55 [-1.53 to 0.43] p = 0.25) and TGFβ-secretion (0.68 [-0.39 to 1.76] p = 0.20), BAL and blood cytokine levels were similar for carriage positive volunteers [IFNγ: (0.18 [-0.78 to 1.14] p = 0.69); TGFβ:(-0.04 [-1.48 to 1.40] p = 0.95)]. Again, we note that these observations are against a background of non-significant differences in matched time-point blood $CD4^+IFN\gamma^+$-secretion (FC = 1.90, p = 0.34) and undetectable $CD4^+TGF\beta^+$-secretion (FC = 0.73, p = 0.44) (Figure 5.7, Table 5.4).

Therefore, PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae* differed in the lung and blood: where responses were significant, PspA-specific $CD4^+TNF\alpha^+$ response levels were lower in BAL compared to blood. This further still may reflect the reduced immunodominance of PspA in the lung compared to blood.


(a)
unstimulated
stimulated
% cytokine-secreting CD4+ T-cells (log10)
10
1
0.1
0.01
0.001
0.0001
IL-17A
IL22
TNFα
IFNγ
TGFβ
US
F2
F4
F7
PspA/Rx1
Stimulus

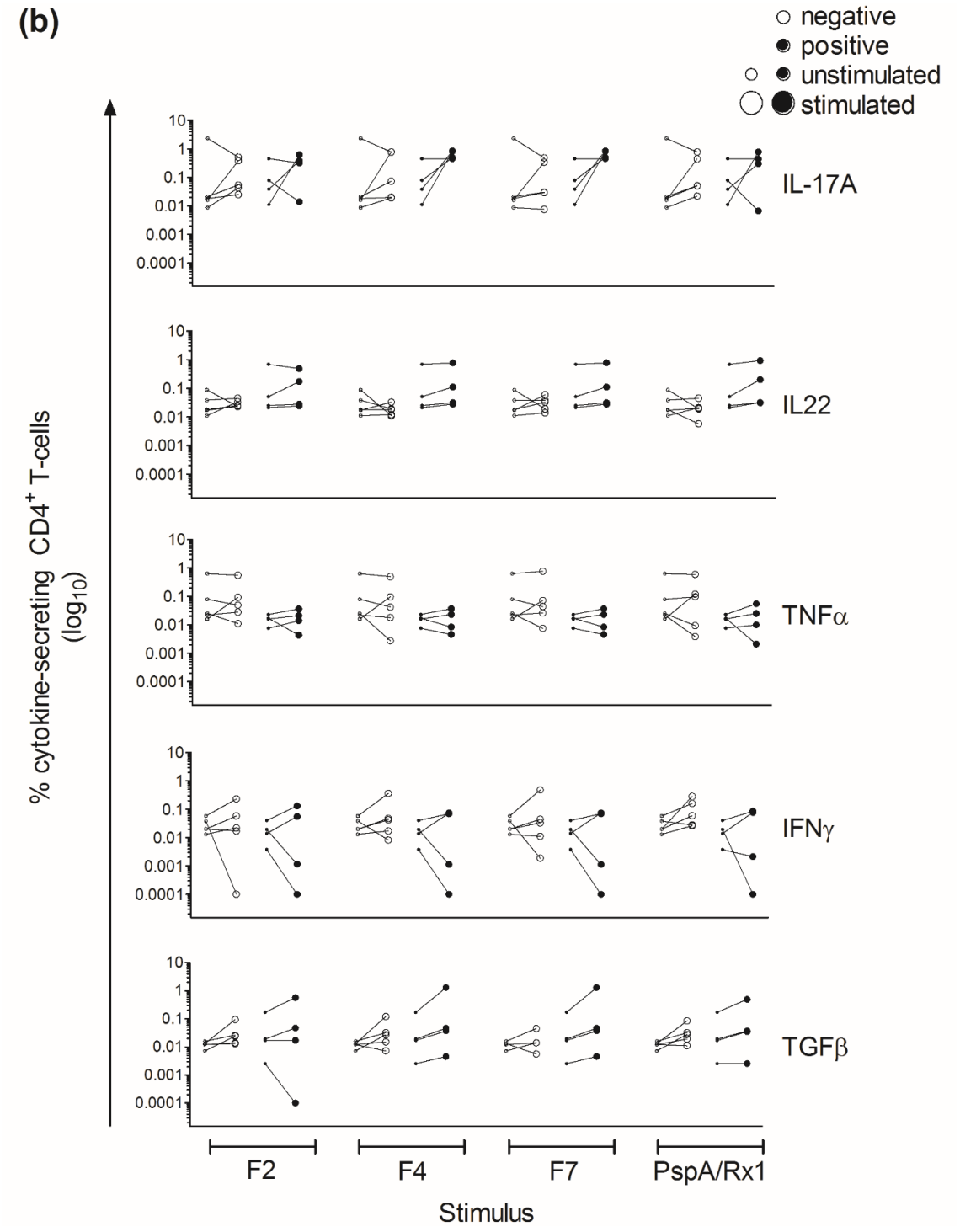


Figure 5.8 Matched BAL time-point blood PspA-specific CD4⁺ T-cell responses to *S. pneumoniae*. CD4⁺ IL-17A, IL22, TNFα, IFNγ and TGFβ secretion in the blood (matched BAL time point) post *S. pneumoniae* inoculation. (a) all volunteers - unstimulated (open circles) and PspA-stimulated (closed circles) (b) by carriage status – carriage negative (open circles), carriage positive (closed circles), unstimulated (small circles), stimulated (large circles). One-way ANOVA with Dunnett's post-hoc corrections (all data $p > 0.05$). Horizontal bars represent mean ± SEM.

5.4 Discussion

Post-inoculation PspA-specific $CD4^+IL\text{-}17A^+$, $IL22^+$, $TNF\alpha^+$ and $IFN\gamma^+$ T-lymphocyte responses to *S. pneumoniae* in the lung did not significantly differ from their unstimulated controls. Two exceptions to this however, were observed on PspA functional helical region-specific responses: pulmonary Fragment 2-specific $CD4^+IL22^+$ and Fragment 4-specific $TNF\alpha^+$ T-cell responses to *S. pneumoniae* were significantly detected. These stimulation parameters were employed as a pilot dataset for exploratory analysis of pre-defined research questions and revealed no effect of carriage and colonisation intensity on PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae*, but revealed reduced levels of F4-specific $CD4^+TNF\alpha$-secretion in BAL compared to blood. All data suggest a non-immunodominant role of PspA in the healthy adult lung, subject to study limitations.

5.4.1 Questions answered: study in field context

The lung has historically been considered as a sterile site. However, it is now known to have its own distinct microbiome, where perturbations to its homeostatic state of microbial entry, elimination and regional growth are thought to contribute to disease susceptibility (Dickson and Huffnagle, 2015).

Pre-exposure to *S. pneumoniae* through nasopharyngeal colonisation results in aspiration into the lungs (Mizgerd, 2012). This leads to the generation of antigen-specific memory T-cells, lung resident and primed to prevent disease on re-infection (Purwar *et al.*, 2011). The presence of lung resident effector memory $CD4^+$ T-cells necessitates measurement of antigen-specific responses *in situ* to accurately characterise recall responses specific to novel protein vaccine candidates in the lung. This may inform pneumococcal vaccine design strategies with a focus on creation of constructs with a directional immune response against pneumococcal pneumonia. Our overall inability to detect a significant pulmonary PspA-specific $CD4^+$ T-cell response suggests PspA is not an immunodominant pulmonary $CD4^+$ T-cell antigen, subject to the limitations discussed.

Previous studies investigating pneumococcal-specific $CD4^+$ T-cell responses in the human lung have similarly to this study used a stimulant concentration of 8 µg/mL, but in place of a single pneumococcal protein antigen, stimulated with a pneumococcal cell culture supernatant (Jambo *et al*., 2011, Sepako *et al*., 2014). Together with our findings, this indicates that PspA of strain Rx1 alone may be of insufficient potency to stimulate a pulmonary $CD4^+$ T-cell response. This supports previous findings in BAL of no induction of anti-PspA (clade 1) IgG on exposure to *S. pneumoniae* serotype 6B (strain BHN418, PspA clade 1) (Wright *et al*., 2012).

Further still, on comparing pilot dataset PspA F4-specific $CD4^+TNF\alpha^+$ T-cell responses in the lung and blood at the same time-point, we observed that cytokine secretion was lower in BAL. These data align with murine studies which have demonstrated at the genetic level, that the virulence of PspA is such that there are reduced levels of *pspa* microRNA (mRNA) expression in the lungs of mice compared to the blood (LeMessurier *et al*., 2006, Oggioni *et al*., 2006), again indicative of the reduced immunodominance of PspA in the lung. This further strengthens our observations of a less prolific role of PspA-immune response in the lung compared to blood.

The human lung is a challenging immune environment: the primary site of pathogen targeting, a delicate balance between sterility and inflammation is crucial to maintain the essential and normal functioning of gaseous exchange. Treg-associated cytokine TGFβ plays a role in both normal lung function, and the pathogenesis of disease. Prevention of lung homeostatic dysregulation includes regulation of local immunomodulation, cell proliferation, differentiation, and control of tissue repair (Bartram and Speer, 2004). Where these processes are perturbed in magnitude or temporally, there is a risk of development of lung pathology with fibrosis: (Neill *et al*., 2012) demonstrated that mice with high levels of TGF-β1-associated with a T-regulatory phenotype were less likely to develop pneumococcal pneumonia. It is therefore conceivable as to why non-PspA-specific $CD4^+TGF\beta^+$ responses were elevated in carriage, reciprocally so to the $CD4^+$IL-$17A^+$ responses, although not significantly different to carriage negative volunteers: this may reflect the regulation of an inflammatory response in a population at increased risk of lung injury, on aspiration of pneumococci during carriage to the lung. This observation aligns with pneumococcal-specific observations of a reciprocal relationship

between T-regulatory and Th17 cell activity in pneumococcal infection at a mucosal site, where Tregs are more abundant in adenoids of children positive for pneumococcal carriage compared to those negative for carriage (Jiang *et al*., 2015, Mubarak *et al*., 2016).

5.4.2 Questions raised: study limitations

Our observation of a global non-robust nature of pulmonary $CD4^+$ T-cell responses post-inoculation points towards PspA as non-immunodominant in long-lived $CD4^+$ T-cell recall responses in the lung, or methodological limitations of sampling.

BAL and matched blood samples were taken an average of 46 days later than day 21 post-inoculation blood samples, therefore it is possible recall responses were dampened and consequently not robust. This finding is in line with the rationale for administration of vaccine boosters to maintain memory populations on depletion of effector T-cell populations. It has been described as the antigen-dependent maintenance of T-cell memory (Gray and Matzinger, 1991), where re-exposure/extended exposure results in more robust $CD4^+$ recall responses (Ravkov and Williams, 2009).

Furthermore, memory responses to *S. pneumoniae* are best investigated in a longitudinal manner so that a time course of immune response can be followed. However, we have observed previously that the nature of a bronchoscopy and lavage induces a pro-inflammatory response which may perturb carriage acquisition rates (Wright *et al*., 2012). Therefore, repetitive lung sampling would not provide an accurate reflection of pulmonary recall cellular responses to the pneumococcus as BAL may prematurely clear carriage. In light of the restrictions on single time-point sampling, it is feasible the assay may respond to further optimisation, so we assess the conditions of the *ex vivo* intracellular cytokine staining assay.

The limited availability of non-adherent cells for antigen stimulation (predominantly lymphocytes), resulted in conducting the BAL intracellular cytokine staining (ICS) assays with at most, half the number of non-adherent cells compared to PBMCs per test condition. Consequently, BAL-derived cell-antigen interactions may have differed from blood-derived PBMCs within a set reaction well. Furthermore, the nature of this mucosal sample (tendency of cells to clump together) resulted in fluctuations in acquisition rate and resultant potential of

more than a single cell passing through the cytometer at once. To maintain quality of single-cell cytokine secretion data, we excluded samples displaying flow cytometry acquisition rate errors, therefore reducing numbers of an already limited dataset.

It is important to note however, that the ICS assay used did not include a $CD4^+$ effector memory marker e.g. CD45RO. Therefore, inferences made on the longevity of proportion of cytokine-secreting PspA-specific $CD4^+$ T-cells, may be masking the specific cell population of interest.

5.4.3 Questions posed: further work and study implications

Our inability to detect a robust PspA-specific pulmonary $CD4^+$ T-cell response to *S. pneumoniae* an average of 67 days post pneumococcal inoculation raises questions as to the role of PspA-specific T-cell memory responses in the adult lung.

The lung is a complex and highly regulated immune environment, so emphasis must be placed on investigating additional immune cell subsets such as alveolar macrophages (AMs). AMs are one of the most abundant cell types in the lung (Balhara and Gounni, 2012), and impaired responses have been associated with pneumonia susceptibility in at risk populations. Burnham and co-workers (2011) have described a relationship between alcohol use disorders and perturbations in gene expression relating AM apoptosis, Wang *et al*. (2008) impaired AM pattern recognition and *S. pneumoniae* clearance amongst morphine users, whereas no association was found between HIV and impaired AM phagocytic activity (Gordon *et al*., 2001). Of note is the demonstrated compartmentalisation of effector memory T-cell responses to *S. pneumoniae* (Jambo *et al*., 2011); this highlights the need to sample and assess cellular responses with a potential protective role against pneumonia, directly from lung and its surrounding tissue.

Investigating pulmonary-specific immune responses to multiple widely expressed and immunogenic pneumococcal proteins, may reveal responses specific to other vaccine candidates: this causes us to re-visit the need to investigate multiple protein vaccine candidates. Nonetheless, the potential to dissect cellular responses elicited in the lung and

correlating experimental human pneumococcal carriage density and duration holds the prospect of aiding tissue-specific vaccine design and merits further study.

CHAPTER 6

Systemic *S. pneumoniae* epitope-induced lymphoproliferation

6 Systemic *S. pneumoniae* epitope-induced lymphoproliferation

6.1 Introduction

Current pneumococcal vaccine strategies target the variable surface polysaccharide of which there are over 90 serotypes: this has led to serotype replacement and vaccine escape. New approaches targeting virulence factors widely expressed across pneumococcal strains and of functional importance, would act to broadly target *S. pneumoniae* strains at a cost to fitness (Cobey and Lipsitch, 2012), hindering the mechanisms they use to survive and proliferate. Targeting multiple conserved virulence factors simultaneously is a strategy with the potential to avoid rapid vaccine escape, slowing vaccine-induced pneumococcal evolution and therefore the selective pressure now so globally prevalent as a result of polysaccharide-based vaccine introduction.

Emerging pneumococcal vaccine strategies focus on immunisation eliciting broad protection across strains (Miyaji *et al*., 2013). One way to obtain broad coverage is to incorporate multiple widely expressed *S. pneumoniae* proteins. Leading candidates are conserved in nature and have demonstrated immunity against pneumococcal infection. Table 1.1 (Chapter 1, Section 1.4.2.2) lists well-characterised candidates alongside their associated functions, and include the six top protein candidates tested in this study:

- Pneumolysin (Ply): a 57 kDa thiol-activated cytolysin. Plays a key role in pneumococcal virulence, primarily by binding cholesterol, oligomerising, and forming large pores in eukaryotic cell membranes resulting in cell death (Steinfort *et al*., 1989, Ratner *et al*., 2006). Ply has been shown to contribute to pneumococcal pneumonia pathogenicity by promoting inflammation, impairing ciliary function and increasing alveolar permeability, in turn decreasing effective gaseous exchange (Mitchell and Dalziel, 2014).
- Pneumococcal surface adhesin A (PsaA): a 37 kDa lipoprotein that plays a key role in virulence by mediating *S. pneumoniae* attachment to and colonisation of host cells (Berry and Paton, 1996). PsaA also acts to evade the host defence by binding

lactoferrin, competitively interfering with apolactoferrin deposition, and preventing host complement deposition (Shaper *et al*., 2004).

- Plasmin and fibronectin binding protein A (PfbA): a 66 kDa polypeptide which exerts its virulence by binding to and invading host cells (Yamaguchi *et al*., 2008).
- Choline-binding proteins:
    - Pneumococcal surface protein C (PspC): a 75 kDa protein which mediates both pneumococcal mucosal epithelial cell adherence to enable invasion, and evades clearance by binding host immune factors including complement factor H, C3 and secretory IgA (Dave *et al*., 2001, Kerr *et al*., 2006, Hammerschmidt *et al*., 1997);
    - Pneumococcal choline-binding protein A (PcpA): a 79 kDa surface antigen which plays a role in pneumococcal adhesion and colonisation (Sanchez-Beato *et al*., 1998);
    - Pneumococcal surface protein A (PspA): an 80 kDa protein which inhibits complement deposition and binds lactoferrin to competitively inhibit apolactoferrin binding and killing (Ren *et al*., 2004).

S. pneumoniae-specific $CD4^+$ T-lymphocyte cellular responses are elevated post-pneumococcal exposure (Wright *et al*., 2012) where elicitation of $CD4^+$ T-cell responses requires binding and presentation of epitopes to a Major Histocompatibility Complex (MHC) class II molecule (Murphy and Weaver, 2017). Larger peptides of 13 – 18 amino acids preferentially bind MHC class II molecules over class I, promoting a $CD4^+$ T-cell immune response over a $CD8^+$ response. Our previous work sought to identify protective linear epitopes of a leading vaccine candidate, PspA, by probing both with serum of healthy adults both susceptible to or protected from pneumococcal carriage following nasal inoculation (EHPC model), and of older adults, a population more susceptible to pneumonia (Owugha *et al*., in preparation). We did not identify any linear epitopes differentially recognised in young compared to older adult cohorts, and therefore did not identify any potentially protective pneumococcal epitopes. Therefore, we sought to identify conserved and functionally important linear pneumococcal protein epitopes using a novel *in silico* approach, and test their immunogenicity *ex vivo*.

Coupling informatics approaches to epitope identification with immunological investigations has proven a useful tool for validating initial epitope selection, as predictions do not always directly correlate functional experimental data (Ip *et al*., 2015). A mainstay and initial screening stage to validate by immunogenicity is the wide-ranging lymphoproliferative assay: this enables immunological assessment of antigen-specific responses to informatics-identified epitopes. It is widely acknowledged however, that no single measure of lymphoproliferation captures the totality of the immune response to antigen (Roederer, 2011). Therefore, measures are often evaluated in tandem. Two employed in this study were:

- Precursor Frequency (PF) as a marker of antigen-specific proliferating cell division probability
- Upper generation proliferation index (UGPI) as a measure of true proliferating cell increase in cell number

A sample dataset exemplifying calculations is as below:

Table 6.1 Sample dataset for calculation of Precursor Frequency and Upper Generation Proliferation Index.

Generation	Total cell number	Parent cell number
Parent	4000	4000
2	800	400
3	1000	300
4	2000	300
5	2800	200
6	5200	150
7	5400	80
8	2100	40

PF = parent cell number (∑ generations 3 – 8)/parent cell number (∑ generations 1 - 8)

= 1070 /5470

= 0.20

UGPI = total cell number (∑ generations 3 – 8)/parent cell number (∑ generations 3 – 8)

= 18500 /1070

= 17.29

The potent mitogen *Phaseolus vulgaris* phytohaemagglutinin (PHA) (Quah *et al*., 2007) contains the major subunit leucoagglutinating phytohemagglutinin, PHA-L (Fitches *et al*., 2001, Kumar *et al*., 2013). Frequently used as a positive control in lymphoproliferative assays, proliferative responses are monitored by staining of cells and monitoring the proportional decrease in visualised dye with increasing number of generations (Crumpton *et al*., 1975). Carboxyfluorescein succinimidyl ester (CFSE) is widely used as a cell dye due in part to high levels of cell permeability promoting homogeneity of staining, and stable covalent coupling to intracellular molecules enabling long-term tracking of cellular dynamics. Together, this allows for monitoring of cell division due to the reliable progressive halving in fluorescence intensity after up to an average of eight successive daughter generation divisions (Parish, 1999).

Using a novel *in silico* computation method of selecting globally conserved peptide sequences of functional importance (fragility islands) (Bright *et al.*, unpublished data), we aimed to identify pneumococcal protein targets with a cost to pathogen fitness, and assess their lymphoproliferative potential. We specifically set out to:

1. Identify the most promising pneumococcal protein vaccine candidates by literature search
2. Identify conserved peptide sequences of functional importance (fragility islands) within selected vaccine candidates, and fragment into 20 aa epitopes
3. Optimise lymphoproliferation assay conditions
4. Conduct the lymphoproliferation assay with single epitopes to select the 20 most proliferative.

Ultimately, down-selection of immunogenic epitopes would go towards identification of a variety of epitopes inducing antigen-specific lymphocyte proliferation in healthy adults, and be fused to create novel protein constructs to test protective potential in murine models of pneumococcal infection.

6.2 Materials and methods

6.2.1 Pneumococcal protein vaccine candidate identification

A literature search was conducted to identify the 30 most promising pneumococcal protein vaccine candidates.

6.2.2 Protein sequence identification and epitope generation

An *in silico* approach to identifying regions of conserved amino acid sequence and functional relevance to *S. pneumoniae* (fragility islands), was taken to listed pneumococcal proteins (Table 1.1, Section 1.4.2.2). Briefly, an algorithm was developed (Blight *et al*., unpublished data) using Java SE Development Kit 8 software (Oracle) in which full length protein sequences were collected and queried for taxonomic identification using NCBI's Entrez protein database (McEntyre, 1998) and Clustal Omega's command line interface binary v.1.2.1 (Sievers *et al*., 2011). Resultant sequences were aligned and conservation levels assessed using a sliding window approach. A conservation value between 0 and 1 was calculated for each window, and adjustments made to account for sequence collection bias. Fragility islands were defined as sequential windows with a conservation value deviating no more than 20% (window size = 15 amino acids; SD of conservation for fragility island $\leqq$ 20%).

Fragility island sequences were fragmented into 20 amino acid (aa) epitopes to promote MHC class II binding, and sequential sequences overlapped by 10 aa to prevent truncation of potentially immunogenic epitopes at fragment junctions. Resultant epitopes were synthesised: peptide purity and quality was assessed by high performance liquid chromatography and mass spectrometry, routinely scoring above 95% (Mimotopes, Clayton Victoria, Australia).

6.2.3 Volunteer recruitment, ethical approval, sample collection and processing

Healthy adult volunteers were recruited under ethical approval granted by The North West 3 Research Ethics Committee, Liverpool East (REC 11/H1002/9) as previously described (Section 2.1.2). Samples were collected and processed as previously described (Section 2.3.3). Briefly, donated heparinised venous blood samples were collected and peripheral blood

mononuclear cells (PBMCs) isolated by density-mediated centrifugation using Ficoll-Paque PLUS (GEHealthcare, Uppsala (Sweden)) according to the manufacturer's instructions. Cells were washed twice in HBSS, re-suspended in RPMI + 20 mM L-glutamine supplemented with antibiotics (penicillin 40 U/mL, streptomycin 40 µg/mL, neomycin 80 µg/mL), seeded into a 24-well plate in a volume of 1 mL and concentration of 1 x 10^6 PBMCs/mL and rested over night at 37°C, 5% CO_2.

6.2.4 Proliferation assay

The potent lectin mitogen phytohaemagglutinin-L or leucoagglutinin (PHA-L), was used as a positive control and in optimising the lymphoproliferation assay. Volunteers (n = 5) were screened for a detectable PHA-L response, and the 2 highest responders selected for epitope-induced lymphocyte proliferation testing.

In optimisation experiments, rested PBMCs were stained with 1.0, 2.5 and 5.0 mM/test CFSE and stimulated with 1.0, 2.5 and 5 µg/test PHA-L in triplicate for 72, 120 and 168 hours. Optimal conditions selected were 2.5 mM CFSE staining, 1.0 µg PHA-L stimulation, and 120 hours duration. Using these conditions, two randomly selected epitopes (epitopes 36 and 47) were titrated for optimisation of stimulant concentration: concentrations of 0.1, 0.5, 1.0, 2.5, 5.0 and 10.0 µg/test were evaluated, and 10.0 µg/test selected.

In epitope-induced proliferation experiments, rested PBMCs were collected and stained with 2.5 mM carboxyfluorescein succinimidyl ester (CFSE) at a concentration of 1 x 10^7 PBMCs/mL and incubated in the dark with shaking (200 rpm) for 20 minutes. PBMCs were subsequently seeded into a 96-well plate in a volume of 200 µL, left unstimulated as a negative control, stimulated with 10 µg of epitope, or 1.0 µg of PHA-L positive control, and incubated at 37°C, 5% CO_2 for 120 hours.

Cells were harvested and proliferation detected by fluorescence. 300,000 cell events were acquired using a BD LSRII Flow Cytometer (Becton Dickinson) and viewed using FacsDiva version 6.1 (BD Biosciences) software in the LSTM Flow Cytometry Facility. ModFit LT v.4.1 was harnessed for proliferation analysis.

6.2.5 Statistical Analysis

Lymphoproliferation in optimisation experiments was assessed using the parameter of Proliferation index (PI), and computed using FlowJo v7.65:

PI = total number of divisions (∑ generations 2 - 7)/parent cell number (∑ generations 2 - 7)

Lymphoproliferation in the subsequent epitope-induced lymphoproliferation pilot study was assessed via Precursor frequency (PF) and upper generation proliferation index (UGPI) computed using the ModFit LT v.4.1 software:

PF = parent cell number (∑ generations 3 - 8)/parent cell number (∑ generations 1 - 8)

UGPI = total cell number (∑ generations 3 - 8)/parent cell number (∑ generations 3 - 8)

Descriptive measures for non-parametric data analysis were employed to analyse epitope-induced proliferation conducted in triplicate, namely median and percentage values.

6.3 Results

6.3.1 Identification of pneumococcal protein vaccine candidates

We conducted a literature search of widely expressed immunogenic proteins with roles in pneumococcal virulence and identified leading vaccine candidates: surface proteins (1 - 18) and enzymes (18 - 30):

1. Pneumococcal surface protein A
2. Pneumococcal surface protein C
3. Pneumococcal surface adhesin A
4. Choline-binding protein A
5. Polyhistidine triad proteins D & E
6. Pneumococcal serine-rich repeat protein
7. Pneumococcal choline-binding protein A
8. Pneumococcal transformation pilus
9. Pneumococcal pilus 1 proteins (RrgA & B)
10. Pneumococcal pilus 2
11. Polyamine transporter D
12. Pneumococcal adhesion and virulence protein A & B
13. Plasmin and fibronectin binding protein A
14. Pneumococcal iron acquisition/uptake protein A
15. Maltose/maltodextrin-binding protein
16. ABC transporter, substrate-binding protein
17. Hypothetical surface exposed protein
18. Cell wall-associated serine protease
19. Pneumolysin
20. IgA1 protease
21. Endonuclease A
22. Neuraminidases A & B
23. Glycosyl hydrolase 25 relating to invasion proteins
24. Hyaluronate lyase

25. Pneumococcal protein endopeptidase O
26. Large zinc metalloproteinase
27. Autolysins B & C
28. Protein for cell wall separation of group B streptococcus
29. Serine-threonine protein kinase
30. α-glycerophosphate oxidase

We therefore identified the thirty most promising pneumococcal vaccine candidates for downstream epitope generation.

6.3.2 Identification of conserved peptide sequences within fragility islands and fragmentation into epitopes

Based upon protein consensus sequence availability and quality of sequence alignments, we down-selected 14 of the original 30 pneumococcal proteins for further analysis. We subsequently identified 36 sequences both of a conserved nature and of functional importance (fragility islands) from the 14 selected proteins (Appendix A).

To promote downstream optimal binding of epitopes to MHC class II molecules in lymphoproliferation assays, we fragmented fragility islands into 20 amino acid (aa) epitopes (where the identified fragility island sequence was 20 aa or longer): we constructed 151 epitope sequences in total. To ensure potential immunogenic linear epitopes were not truncated at fragment junctions, we defined epitopes to overlap by 10 aa (Appendix A). Epitopes not in fragility islands were designated highly diverse controls (HDCs).

On sequence alignment of choline-binding proteins PspC, PcpA and PspA, we discovered that multiple sequence entries had been misnamed. To clearly distinguish between proteins, we re-aligned sequences and grouped them based on clades as revealed by cladistic analysis (Blight *et al*., unpublished data). Figure 6.1 illustrates the distinct branch formation on phylogenetic tree representation, and the three distinct groups (clades 1 - 3) of the newly designated protein 1_2_10: clade 3 divided into two further sub-clades (clades 3.1 and 3.2).

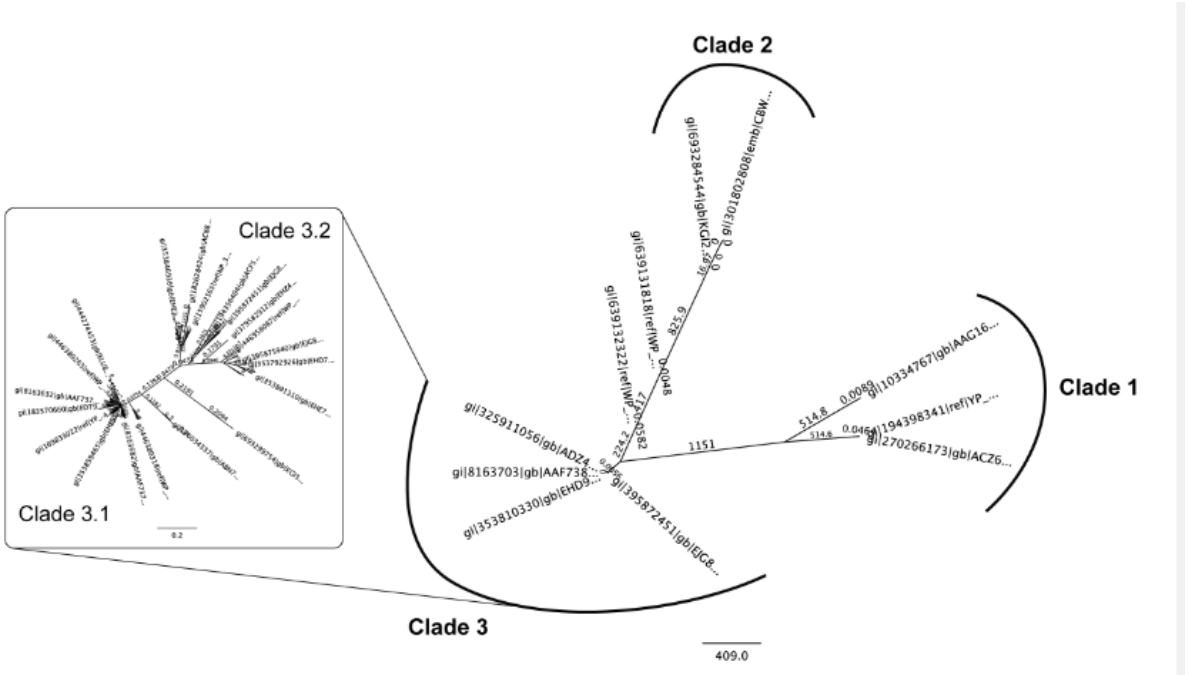


Figure 6.1 Radial cladogram of newly designated protein 1_2_10 illustrating sequence analysis to genetically distinguish between choline-binding proteins labelled PspC, PcpA and PspA. Sequences were aligned and grouped into distinct clades 1 - 3. Clade 3 was further divided into two sub-clades 3.1 and 3.2. Bar represents unit of genetic change.

We removed sub-clade 3.1 from further analysis as domains of functional relevance from constituent sequences were diverse. Sub-clade 3.2 was further divided into clades 3.2.1 and 3.2.2: clade 3.2.1 contained key fragility islands which were conserved (translocated promotor, TPR, and TATA-box regions), whereas clade 3.2.2 did not contain any islands of conservation. Furthermore, sub-clades 3.1 and 3.2.2 were more closely related to clades 1 and 2 compared to sub-clade 3.2.1, therefore we selected clade 3.2.1 as a distinct representative of clade 3 choline binding proteins (Blight *et al*., unpublished data).

We therefore identified conserved peptide sequences of functional importance (fragility islands) within selected vaccine candidates, and fragmented them into 20 aa linear epitopes. For this pilot study we selected epitopes listed 1 - 55 spanning 6 proteins to investigate pneumococcal epitope-induced lymphoproliferation in healthy adults (Appendix A).

6.3.3 Optimisation of lymphoproliferation assay conditions

Due to the heterogeneity in volunteer responses, we selected 2 volunteers with the highest lymphoproliferation induced by positive control PHA-L, from an original screen of 5. This was to ensure we were working with detectable PHA-L responses for the optimisation of assay conditions. We tested PHA-L stimulation at 1.0, 2.5 and 5 µg/test, CFSE concentrations of 1.0, 2.5 and 5.0 mM/test, assay durations of 72, 120 and 168 hours, and pneumococcal epitope concentrations of 0.1, 0.5, 1.0, 2.5 5.0 and 10.0 µg/test.

We measured the greatest lymphocyte proliferation on stimulation of PBMCs with 1.0 µg/test of PHA-L (24.6% lymphocytes in total cell population): cell proliferation on stimulation with 2.5 µg/test resulted in a 4-fold ±1.67 decrease in lymphocyte abundance relative to unstimulated control (10.3% lymphocytes), and stimulation with 5 µg/test a 9-fold ±0.90 loss in cell yield (2.6% lymphocytes) (Figure 6.2). On staining cells with 2.5 mM CFSE/test, we detected the highest resolution of daughter generations giving a proliferation index (PI) of 3.9, compared to 2.8 at 1 mM and 2.7 at 5 mM CFSE (Figure 6.3).

We detected an increase in proliferation indices with duration of stimulation: 1.02 at 72 hours, 1.26 at 120 hours and 2.21 at 168 hours. However, the marked increase in apoptotic lymphocytes at 168 hours resulted in 15% of the cell population identified as lymphocytes compared to 24.6% at 72 hours and 54.3% at 120 hours. We therefore selected 120 hours as the optimal assay duration (Figure 6.4).

After we optimised assay conditions as 2.5 mM CFSE staining, 1.0 µg PHA-L stimulation, and 120 hours duration, we titrated for epitope concentration. Epitopes 36 and 47 were randomly selected and lymphocyte proliferation measured at 0.1, 0.5, 1.0, 2.5 5.0 and 10.0 µg/test (Table 6.2).

We did not detect lymphoproliferation on stimulation of PBMCs with epitope concentrations of 0.1 to 5.0 µg/test: upper generation proliferation indices (UGPI) were below that of the unstimulated control (UGPI < 9.28). However, on stimulation with 10 µg/test, we detected proliferative activity: UGPIs were 15.07 for epitope 36 (background-corrected 5.5), and 12.07 (background-corrected 2.5) for epitope 47. We consequently selected a final epitope assay concentration of 10 µg/test.

Therefore, lymphoproliferation assay conditions were optimised to 2.5 mM CFSE staining, 10 µg epitope and 1 µg PHA-L stimulations over 120 hours duration.

Table 6.2 Upper generation proliferation indices for optimisation of epitope stimulant concentration.

Stimulant concentration (µg/ test)	Epitope 36	Epitope 47
0	9.28	
0.1	8.50	7.70
0.5	8.50	6.92
1.0	8.87	6.73
2.5	8.67	8.22
5.0	7.91	7.75
10.0	15.07	12.07

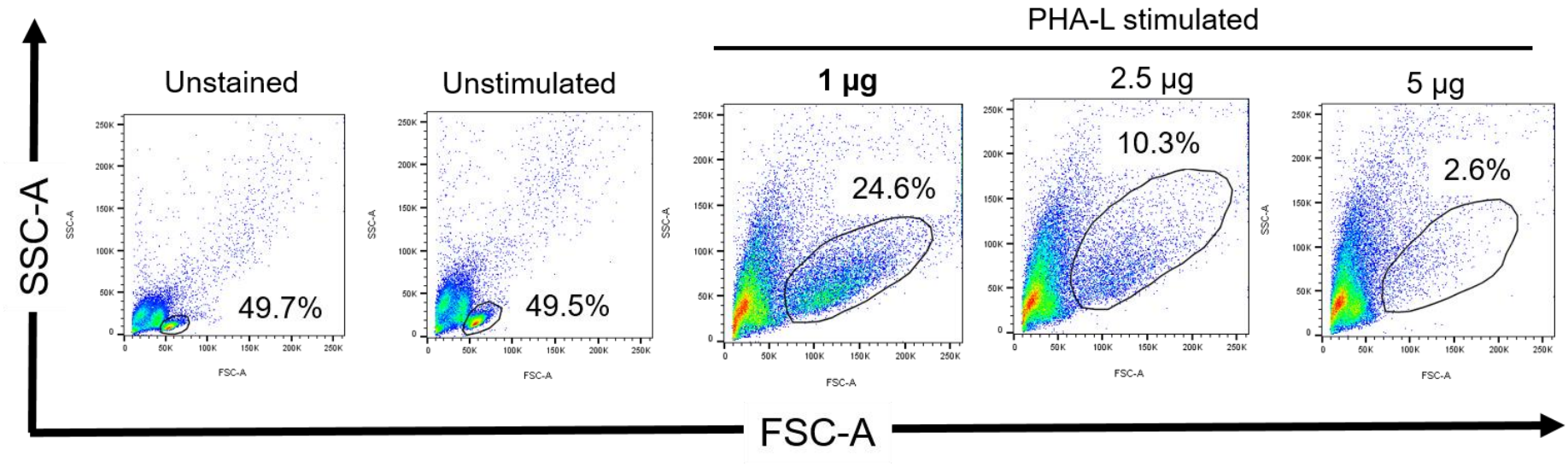


Figure 6.2 Lymphoproliferation assay PHA-L positive control stimulant concentration optimisation. Positive control PHA-L responses were assessed at 1.0, 2.5 and 5 µg/test. Percentage lymphocytes indicated within plot. FSC-A – forward scatter (measure of cell size); SSC-A – side scatter (measure of cell granularity/internal complexity).

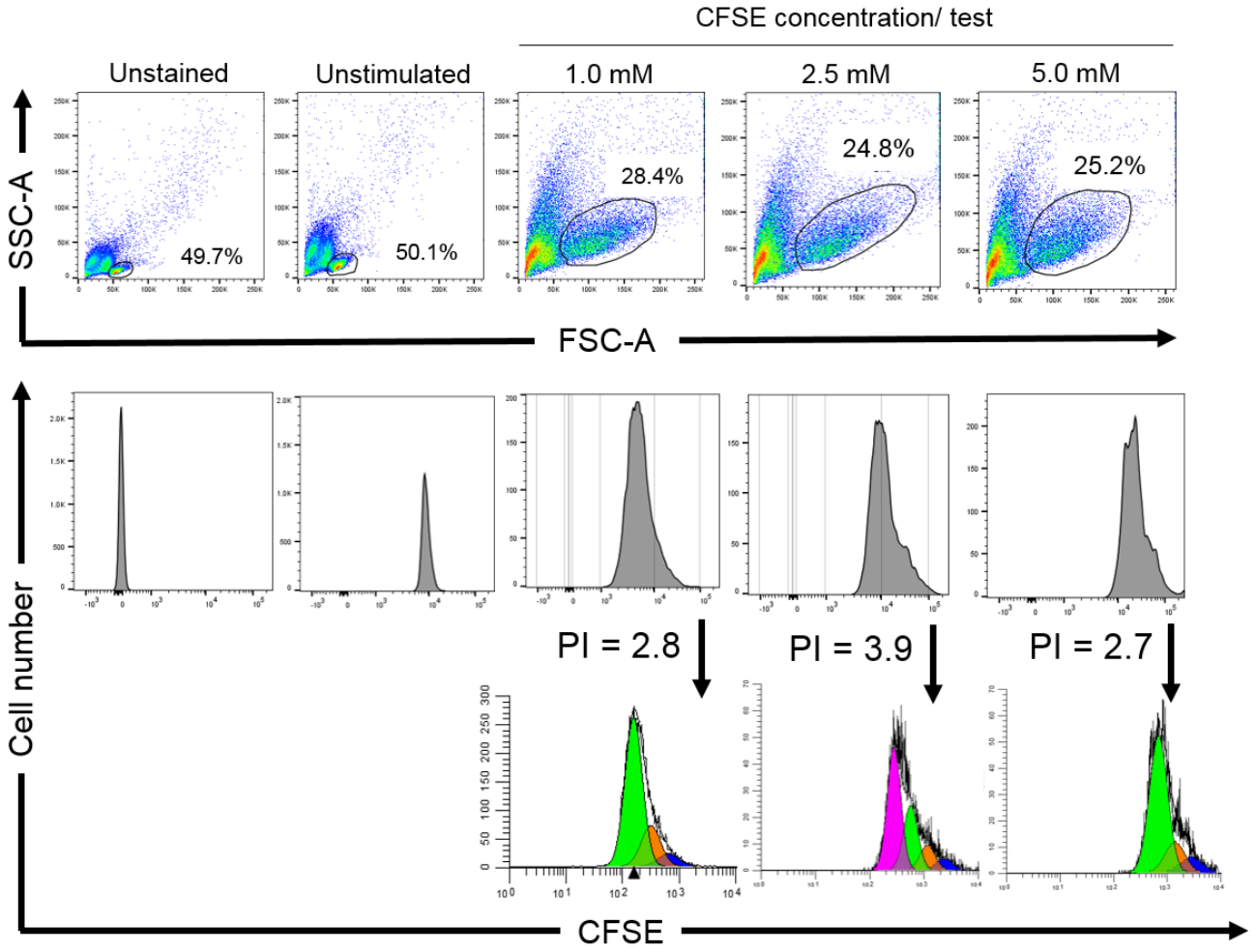


Figure 6.3 Lymphoproliferation assay CFSE concentration optimisation. Positive control PHA-L responses at 1.0 µg/test used to titrate CFSE concentration. Percentage lymphocytes indicated within each dot plot. Histograms demonstrate daughter generation by colour, and Proliferation Index (PI) per CFSE dilution. FSC-A – forward scatter (measure of cell size); SSC-A – side scatter (measure of cell granularity/internal complexity); CFSE - carboxyfluorescein succinimidyl ester (lymphocyte stain).

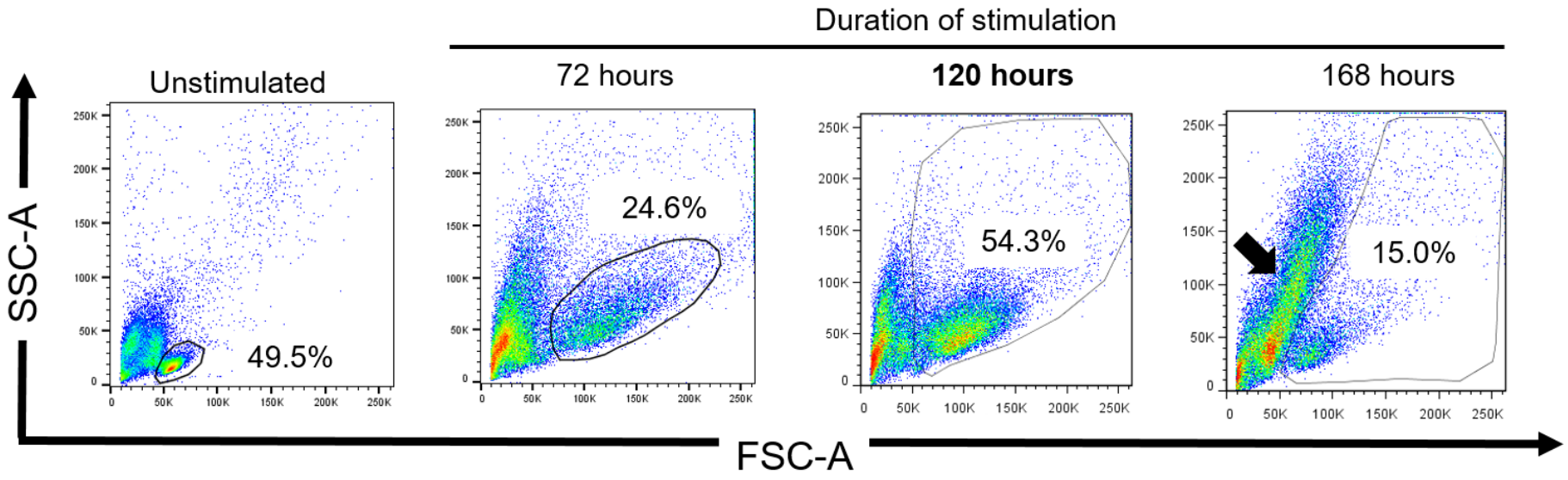


Figure 6.4 Lymphoproliferation assay duration optimisation conditions. Positive control PHA-L proliferation potential assessed at 72, 120 and 168 hours. Percentage lymphocytes indicated within plot. Bold arrow denotes region of high lymphocyte apoptosis. Unstimulated and stimulated lymphocyte. FSC-A – forward scatter (measure of cell size); SSC-A – side scatter (measure of cell granularity/internal complexity).

6.3.4 Selection of lymphoproliferative epitopes

Immunogenic proteins in existing vaccines such as the tetanus toxoid (TT), are large and in the region of 150 kDa (Paoletti *et al*., 1990). This translates to > 1,300 amino acids and therefore a multiple-epitope protein made up of 20 aa fragility island epitopes could include up to sixty in number. As this pilot study measured lymphoproliferative responses in one third of an available 151 epitopes (n = 55), we evaluated the top 10 ranked epitope-induced lymphoproliferative responses in two volunteers (total n = 20), for selection towards a multiple-epitope protein of a final size similar to the TT (total n = 60 epitopes).

Our human adult volunteers elicited a range of recall proliferative responses to pneumococcal protein epitopes (Figure 6.4). Therefore, an epitope hit was defined as concurrent precursor frequency (PF) and upper generation proliferation index (UGPI) rankings within the twenty ranked most proliferative (Appendix B). The top ten epitopes per volunteer were selected to constitute the twenty suitable for down-selection, namely:

- Volunteer 1: epitopes 1, 2, 3, 4, 37, 38, 44, 45, 50, 52 and 54
- Volunteer 2: epitopes 14, 16, 18, 19, 20, 21, 22, 23, 24 and 25.

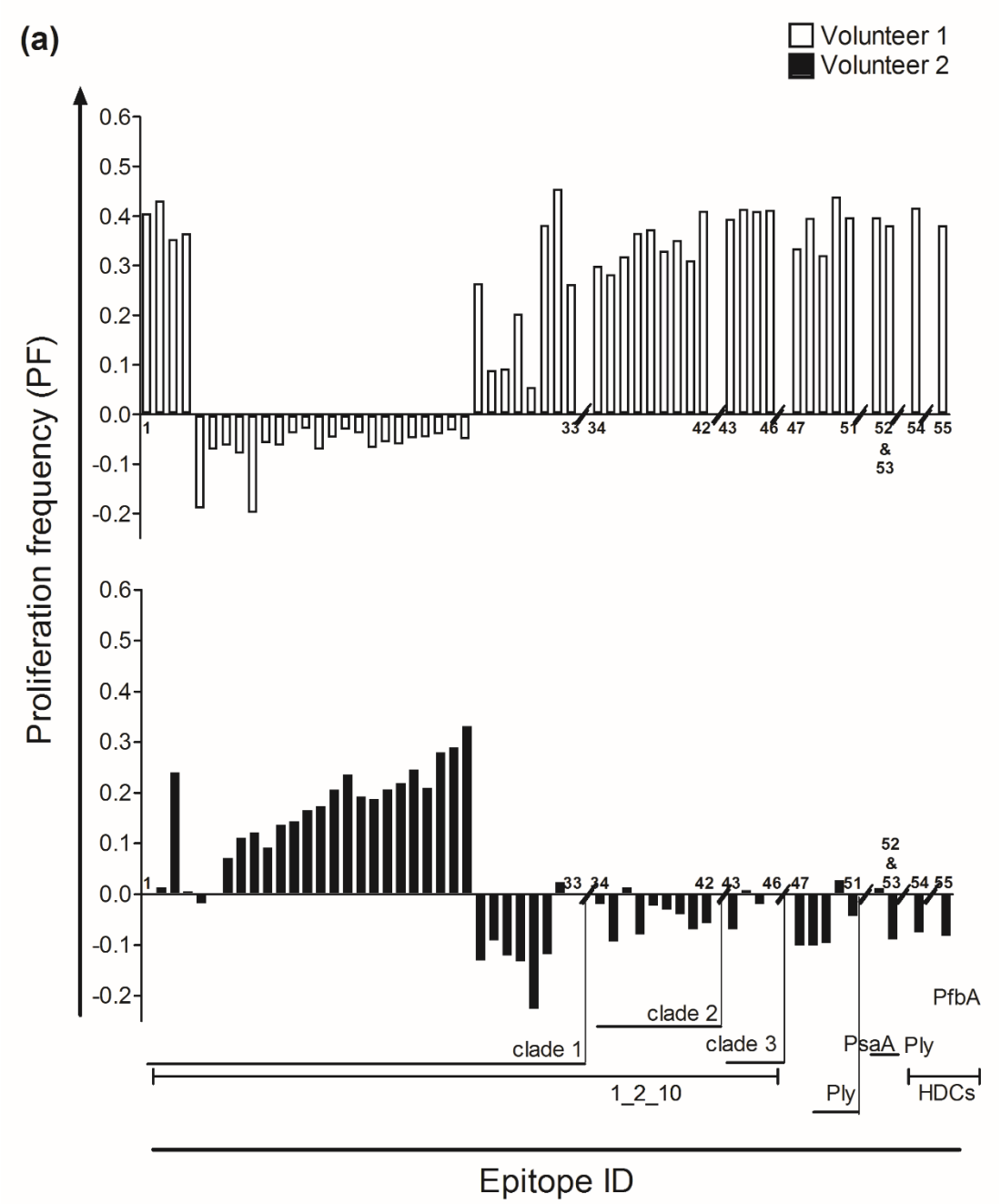
Therefore, we selected the 20 most proliferative epitopes from a lymphoproliferation assay in a pilot of 55 epitopes.

6.3.5 Lymphoproliferative epitopes

On evaluating the top 20 ranked epitope-induced lymphoproliferative response rankings, epitope 3 of protein 1_2_10 clade 1 induced the highest cross-volunteer rankings of PF and UGPI (Appendix B).

Epitope 3 stimulated PF responses ranking 20 in volunteer 1 and 5 in volunteer 2, where UGPI responses were 5 in volunteer 1 (54 in volunteer 2). This was closely followed by epitope 4 PF rankings of 19 for volunteer 1, and 27 for volunteer 2: UGPI rankings were 7 and 20 for volunteers 1 and 2 respectively.

Epitopes 3 and 4 originated from consecutive regions of the same protein (choline binding protein 1_2_10 clade 1), therefore their consecutive high rankings in lymphoproliferation identified a highly immunogenic fragility island region.


(a)
Volunteer 1
Volunteer 2
Proliferation frequency (PF)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
1
33 34
42 43
46 47
51
52 & 53
54 55
clade 1
clade 2
clade 3
PfbA
PsaA
Ply
1_2_10
Ply
HDCs
Epitope ID

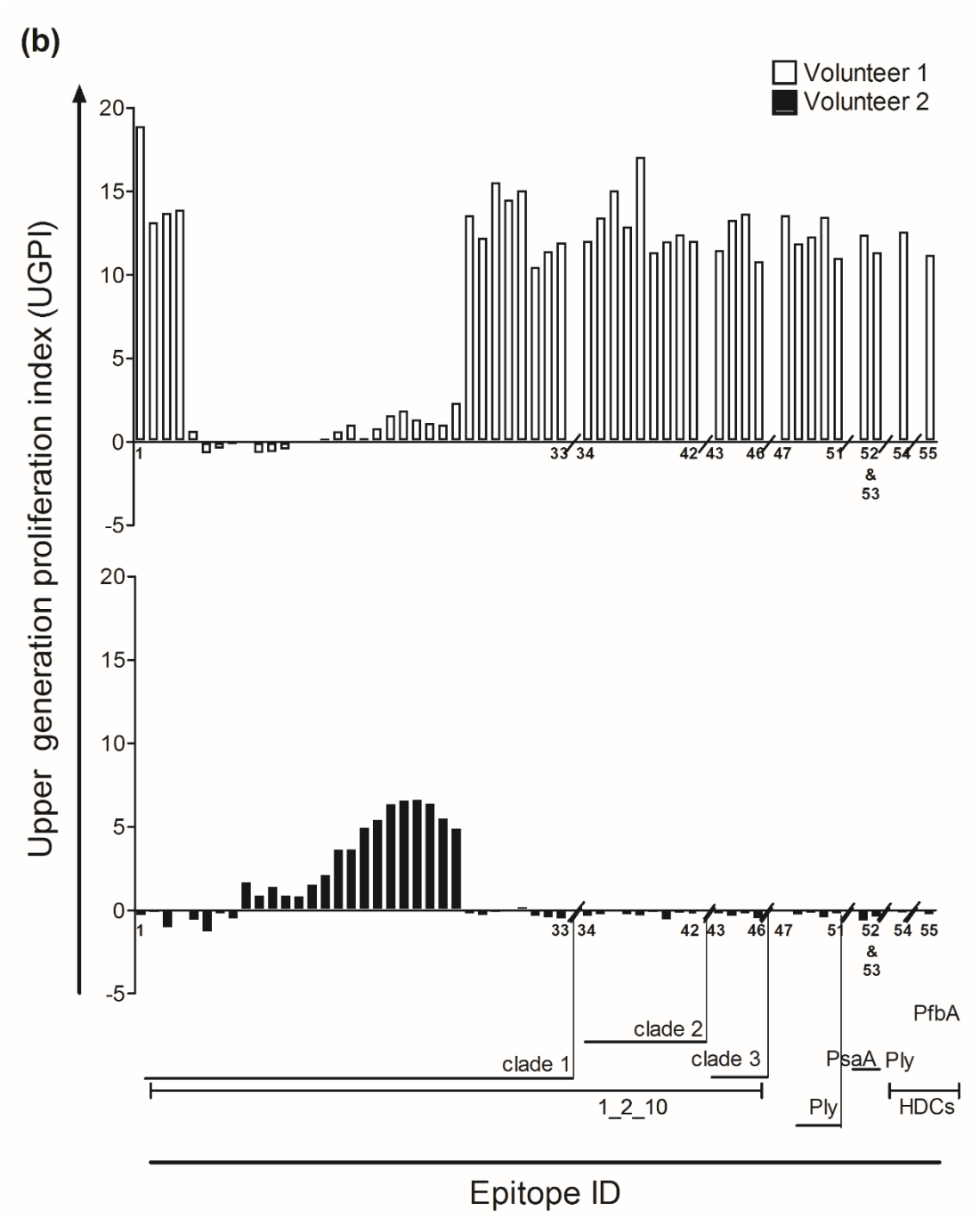


Figure 6.5 Distribution of antigen-specific lymphoproliferative responses to pneumococcal protein epitopes. Unstimulated-corrected values presented for volunteer 1 (open bars) and volunteer 2 (filled bars) where (a) Precursor frequency (PF) probability of each cell dividing at least once (range; 0 to 1) (b) Upper generation proliferation index (UGPI): fold expansion of antigen-specific responding cells (proliferation index at generation 3 and above). Parent proteins from which epitopes derived indicated on the x-axis. HDCs – highly diverse controls, Ply – pneumolysin, PsaA – pneumococcal surface adhesion A, PfbA – plasmin and fibronectin binding protein A.

6.3.6 Lymphoproliferative protein identification

Percentage epitope responses per parent protein were assessed to identify lymphoproliferative proteins. Where both PF and UGPI measurements were above the median parameter value, responses were defined as a positive. Volunteers 1 and 2 mounted an antigen-specific response to test epitopes from all proteins excluding PsaA in volunteer 2 (PF: 50%, UGPI: 0%) and PfbA HDC responses in both volunteers (volunteer 1: UGPI = 0%; volunteer 2: PF = 0%) (Figure 6.5). This result indicated HDC PfbA epitope 55 as a pneumococcal-specific negative control for lymphoproliferative assays. The alternative HDC however, Ply epitope 54, induced a positive response in both volunteers (PF and UGPI = 100%).

The choline binding protein epitopes induced the greatest proportion of responses in both volunteers 1 and 2. Volunteer 1 responses were greatest to clades 2 (PF: 100%; UGPI: 89%) and 3.2.1 (PF: 100%; UGPI: 75%), whereas volunteer 2 responded highest to clade 1 epitopes (PF: 73%; UGPI: 67%) (Figure 6.5).

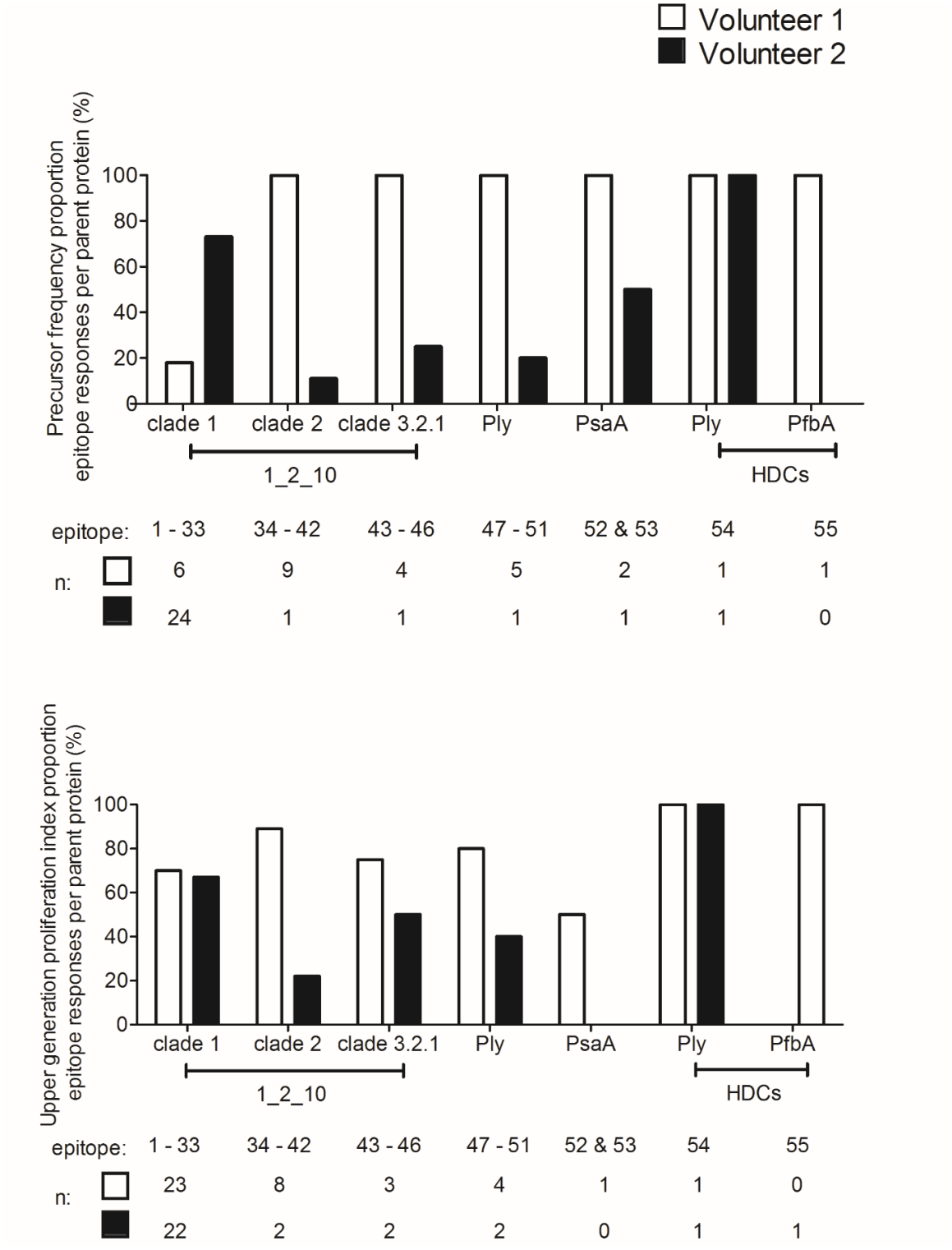


Figure 6.6 Percentage epitope response per parent protein. Proportion of protein epitopes with precursor frequency (top) or upper generation proliferation index (bottom) values above the median values graphed. Protein IDs on x-axis, with epitopes derived below. Number of lymphoproliferation-inducing epitopes (n) for volunteer 1 (open bars) and volunteer 2 (closed bars). HDC: highly diverse control; 1_2_10: choline binding protein 1_2_10. HDCs – highly diverse controls, Ply – pneumolysin, PsaA – pneumococcal surface adhesion A, PfbA – plasmin and fibronectin binding protein A.

6.4 Discussion

We optimised an assay for detection of *S. pneumoniae* protein linear epitope-induced lymphoproliferation and demonstrated that epitope 3 of in-study defined choline-binding protein 1_2_10 clade 1 induced the greatest proliferative activity in selected healthy adults. In this pilot study, lymphoproliferation was measured by precursor frequency (probability of antigen-specific proliferating cell division) and upper generation proliferation index (measure of increase in cell number of true proliferating cells). We also identified a pneumococcal-specific highly diverse control linear epitope, number 55, originating from pneumococcal plasmin and fibronectin protein A (PfbA).

6.4.1 Questions answered: study in field context

The majority of pneumococcal vaccine candidates employed in this study were cell surface proteins, chosen due to studies in the field demonstrating them as protective against pneumococcal infection. Exposed to the host immune system on invasion, these polypeptides are a common target in vaccine design (Gamez and Hammerschmidt, 2012). Our *in silico* identification of a PsaA fragility island located within the zinc-uptake component (epitopes 52 and 53), and its ability to induce lymphocyte proliferation, aligns with the findings of Lagousi and colleagues (2015). Their approach to identifying 10 immunodominant B-cell epitopes in patients with IPD yielded 10 immunodominant epitopes, 4 of which were within the zinc finger region.

A reoccurring motif falling within our identified fragility islands and from which we selected conserved sequences, was the leucine rich repeat (LRR). These sequences form alternating alpha helix-beta strand motifs, which in succession build a ‘horse-shoe’-shaped molecule common on cell surface proteins, and play a role in providing a scaffold for protein-protein interactions necessary for host cell adherence (Kobe and Kajava, 2001, Glover *et al*., 2008).

It is widely acknowledged that computational methods of epitope identification must be coupled with immunological techniques for maximum potential (Ip *et al*., 2015). We employed lymphoproliferation assays to compare proliferative potential of epitopes in two healthy adult

volunteers. Newly categorised choline binding protein 1_2_10 clade 1 (closest consensus homology to PspC) yielded epitopes with highly variant proliferation profiles in both volunteers. PspC is a polymorphic protein and is known to generate antibodies cross-reactive to choline binding protein PspA (Brooks-Walter *et al*., 1999). Furthermore, PspA has been shown to contain epitopes eliciting protection against infection (Daniels *et al*., 2010), and we have described PspA B-cell epitopes as a marker of pneumococcal exposure in adults (Owugha *et al*., in preparation), noting previous reports of PspA as a non-immunodominant protein antigen in paediatric cases of IPD (Lagousi *et al*., 2015).

Our method of identifying conserved epitopes of functional importance is novel in its incorporation and weighting of global coverage of protein sequences. This accounts for any collection bias and therefore provides a truer reflection of global distribution and utility as a vaccine target (Blight *et al*., unpublished data). Chandra and Yadav (2016) acknowledge the importance of population bias in their identification of *Mycobacterium immunogenum* antigens, but we have utilised an algorithm described for the first time which integrates this factor into epitope discovery.

6.4.2 Questions raised: study limitations

Selecting virulence proteins to investigate based on prior studies promoting antigens as promising vaccine candidates poses its limitations. Proteins that may not play a major role in pneumococcal virulence but be highly expressed and therefore serve as an effective pathogen targeting mechanism, may be overlooked. Furthermore, if epitopes of poorly virulent yet densely expressed proteins were coupled to virulence epitopes, there is prospect of a combined effect of a target both highly expressed with a cost to pneumococcal fitness. Therefore, techniques which employ a non-selective approach to screening of the pneumococcal proteome to identify such peptides are favourable. An overview of high throughput T-cell epitope mapping techniques in vaccine development (Malherbe, 2009) includes: the mainstay enzyme-linked immunospot assays (ELISPOT) which monitors the cytokine secreting activity of antigen-induced PBMCs; and an alternative flow cytometry

method known as tetramer-guided epitope mapping, where MHC class II tetramers are used to label antigen-stimulated PBMCs to identify antigenic epitopes.

We employed the long-standing lymphoproliferation technique as an initial basic method of screening epitope immunogenicity, harnessing measures of precursor frequency (PF) as a marker of probability that antigen-specific proliferating cells divide at least once, and upper generation proliferation index (UGPI) as a measure of increase in number of true proliferating cells. These two measures were evaluated in tandem because one alone does not give a full picture of lymphoproliferation and may have skewed analysis (Roederer, 2011). We did not incorporate the basic measure of number of epitope-induced daughter generations, as this parameter has the propensity to skew conclusions toward non-lymphoproliferative epitopes where few new generations are being formed. Biologically, this can be attributed to a propensity towards epitope-induced clonal expansion over division, a functional distinction worth consideration. The acquisition of various effector and memory phenotypes which promote functional immune responses would be beneficial in vaccine-induced protection, and is better reflected in parameters of PF and UGPI which incorporate total cell number and therefore the population size.

A further complication to assessing lymphoproliferation responses lies in the variation between human responses. Differences in immune response to the same antigen across volunteers is an expected phenomenon due to the intrinsic host factor of human leucocyte antigen (HLA) variation. We therefore ranked lymphoproliferation per volunteer, and selected 10 different epitopes from each which were in the top twenty listed as no single epitope was cross-volunteer lymphoproliferative (as measured by both top 20 PF and UGPI responses). Studies identifying immunogenic epitopes to other pathogens or allergens have human volunteer sample sets an order of magnitude greater than this pilot - n = 22 when investigating immunogenic *Mycobacterium tuberculosis* epitopes (Panigada *et al*., 2002) and n = 18 for peanut allergen epitope identification (Prickett *et al*., 2013) - yet require an epitope selection cut-off of 50% of volunteers with a positive $CD4^+$ lymphoproliferative response to an epitope.

Furthermore, the exogenous factor of unknown pneumococcal exposure history which affects variation: it is understood that individual responses to pneumococcal proteins vary across

individuals, and that this increases with age and exposure, measurable in infants as young as 6 months but decreasing again in the elderly who have lower rates of colonisation and therefore pneumococcal exposure (Rapola *et al*., 2000). Therefore, exposing our volunteers to a controlled dose of pneumococci in our Experimental Human Pneumococcal Carriage (EHPC) model may serve to boost immune response and enable investigation of epitope responses due to recent exposure, eliciting a more pronounced and therefore comparison of epitope-induced lymphoproliferative response. An increased $CD4^+$ T-cell response to hepatitis delta virus (HDV) was observed in volunteers with signs of inactive HDV-induced disease, compared to control subjects and patients with active disease (Nisini *et al*., 1997).

6.4.3 Questions posed: further work and study implications

Lymphoproliferation was utilised in this pilot study as an initial screen to assess epitope-induced T-cell immunogenicity. To reduce the hurdles of volunteer HLA variation with this technique, computational selection of epitopes that bind promiscuously to MHC II molecules is an approach that has been harnessed to down-select epitopes (Diethelm-Okita *et al*., 1997). Tools exist to aid in their prediction and include the Immune Epitope Database and analysis resource (Vita *et al*., 2010): these aids have been used in selection of immune epitopes for other bacterial pathogens such as *Staphylococcus aureus* (Yu *et al*., 2015) and can be employed to improve future epitope selection screening.

The data presented here represent a pilot dataset for the first stage in investigations to produce a multiple epitope vaccine protective against pneumococcal infection. Results demonstrated the ability to measure epitope-induced lymphoproliferation by PF and UGPI, but highlighted the need for larger datasets due to the variation in human responses.

Going forward, the remaining 86 epitopes will be assayed in a similar manner with a larger sample size of ten volunteers (the pilot epitope dataset will be increased), and epitope pools screened and titrated to identify the optimal combination and concentration of lymphoproliferative epitopes. The method of pooling epitopes is an approach which has been taken to identification of immunogenic epitopes in HIV (Rubio-Infante *et al*., 2015) and hepatitis E (Aggarwal *et al*., 2007). Cytokine analysis of cell culture supernatants harvested from

lymphoproliferative assays can then be conducted to investigate polarisation in T-cell immune responses, and further select an epitope pool secreting cytokines toward a $CD4^+$ T-cell profile.

The end goal this project is the construction of a novel multiple-epitope protein that can be used to immunise mice and test for protection from a range of pneumococcal infections. However, Macmillan and co-workers' (2008) caution on construction of novel proteins must be heralded: they noted that the original ability of whole outer membrane fractions of *Anaplasma marginale* to elicit protection against disease due to complex structural interactions, may be abrogated on fusion of peptides that what would on the bacterial membrane have been distally located epitopes. We aim to decipher the optimal combination of peptides for fusion protein construction by initially investigating lymphoproliferation on stimulation with epitope pools. Epitopes from lymphoproliferative pools will be fused in a variety of sequences to identify the most protective novel protein, and address any potential sub-optimal peptide structural interactions.

CHAPTER 7

General discussion

7 General discussion

Currently licensed vaccines against pneumococcal infection are efficacious against invasive disease syndromes, but less so against mucosal infections including pneumonia. Furthermore, the serotype-targeted approach of pneumococcal conjugate vaccines (PCVs) to vaccination permits *S. pneumoniae* vaccine escape and proliferation of disease by non-vaccine serotypes. Therefore, there is a merited need for research into mucosally effective serotype-independent vaccination strategies against pneumococcal infection.

The Experimental Human Pneumococcal Carriage model has been demonstrated as safe, efficient and cost-effective in testing the efficacy of PCV13 against pneumococcal colonisation. Using this platform, we investigated the relationship between the biological state of carriage as a prerequisite and proxy to infection in the natural host, and pneumococcal protein vaccine candidate immunity, with an emphasis on pneumococcal surface protein A (PspA). We aimed to:

1. Further our current understanding of humoral PspA-specific responses to *S. pneumoniae*
2. Investigate blood PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae*
3. Investigate BAL PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae*
4. Investigate *S. pneumoniae* protein-specific systemic epitope-induced lymphoproliferation

7.1 Questions answered: a summary of thesis findings and implications for new vaccines against pneumonia

7.1.1 Humoral PspA-specific responses to *S. pneumoniae*

We describe for the first time a suggested PspA family-specific protection against re-colonisation with *S. pneumoniae* vaccine serotype 6B, in individuals who had a recent episode of natural carriage. Furthermore, controlled dosage exposure to *S. pneumoniae* induced cross-reactive IgG to homologous and heterologous PspA family types in individuals both

protected from and susceptible to carriage. Post-inoculation PspA-specific IgG binding functionality however, was greatest in carriage positive volunteers.

These data highlight a potential role of PspA in protection from carriage: this observation is family-restricted, and therefore re-iterate the importance of inclusion of PspA family 1 and 2 antigens in serotype-independent vaccine strategies. Humoral-specific observations of promiscuous PspA family binding demonstrates PspA-specific IgG as a marker of pneumococcal exposure, but not associated to prevention of carriage.

7.1.2 Blood PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae*

We have provided the first evidence of measurable $CD4^+$ T-cell responses specific to an individual pneumococcal purified protein in the blood of healthy adults both before and after carriage. Post-inoculation, we observed a boost in PspA-specific (alpha helical domain Fragment 2 and proline-rich region Fragment 7) $CD4^+TNF\alpha^+$ responses to *S. pneumoniae*, and a weak yet significant inverse correlation between PspA-specific $CD4^+TGF\beta^+$ responses and colonisation intensity.

Our observations highlight the role of $CD4^+TNF\alpha^+$ responses to *S. pneumoniae* colonisation as markers of pneumococcal carriage, and $CD4^+TGF\beta^+$ responses as playing a role in control of colonisation in healthy adults. Together, these data demonstrate PspA immunogenicity, and support a PspA vaccine effort in targeting pneumococcal blood infections and diseases.

7.1.3 BAL PspA-specific $CD4^+$ T-cell responses to *S. pneumoniae*

We did not detect a robust PspA-specific $CD4^+$ T-cell response to *S. pneumoniae* in BAL of healthy adults after pneumococcal exposure: levels were reduced compared to those in blood. These data suggest a compartmentalisation in immune response, and non-immunodominant role of PspA in response to pneumococcal exposure in the healthy adult lung. They do not support a PspA vaccine effort in targeting pneumococcal lung infections and diseases including pneumonia (subject to limitations discussed in Chapter 5), therefore merit research of alternative pneumococcal protein-induced immunity in the human lung.

7.1.4 Systemic *S. pneumoniae* epitope-induced lymphoproliferation

In a pilot study of pneumococcal epitope-induced lymphoproliferation, we demonstrated an epitope within the conserved and functionally relevant fragility sequence of newly defined choline-binding protein 1_2_10 clade 1, induced the highest lymphoproliferative activity in healthy adults (Epitope 3). A highly diverse control (Epitope 55) from pneumococcal plasmin and fibronectin protein A (PfbA), was conversely universally poor as a lymphoproliferation inducer. The observation of cross-volunteer immunogenic Epitope 3 and non-immunogenic Epitope 55, showcase the optimisation of an assay with ability to discriminate lymphoproliferation levels on stimulation with 20 aa linear epitopes.

These data preliminarily support the combination of *in silico* computational methods with immunological techniques to identify conserved pneumococcal epitopes of functional importance, and subsequently validate their immunogenicity in efforts towards design of a multiple pneumococcal protein vaccine.

7.2 Conclusions and perspectives

We have optimised a protocol for measurement of pneumococcal protein-specific $CD4^+$ T-lymphocyte responses to *S. pneumoniae* in the blood of healthy adults. With this we demonstrated PspA-specific $CD4^+TNF\alpha^+$ responses to *S. pneumoniae* as markers of carriage, and PspA-specific $CD4^+TGF\beta^+$ responses to *S. pneumoniae* as playing a role in regulation of colonisation in adults susceptible to carriage. We did not detect significant $CD4^+$ T-cell PspA immune responses to *S. pneumoniae* in bronchoalveolar lavage, therefore suggest PspA is not an immunodominant pneumococcal antigen in the lung. Although the protective potential of PspA was emphasised on observing a role of homologous family protection from carriage re-acquisition, it is axiomatic that research into multiple pneumococcal proteins particularly at the relevant mucosal site of the lung, would support serotype independent pneumococcal vaccine design vaccines efforts against pneumococcal pneumonia.

Appendices

Appendix A Fragmentation of conserved sequences within fragility islands

Protein	Epitope ID	Sequence	Amino acids (n)
1_2_10 clade 1	1	KTVPDGAKLTGEAGKAYNET	20
	2	GEAGKAYNETRTYAKEVVDK	20
	3	RTYAKEVVDKSKKLLSQTAV	20
	4	SKKLLSQTAVTMDELAMQLT	20
	5	TMDELAMQLTKLNDAMSKLK	20
	6	KLNDAMSKLKEAKAKLVPEV	20
	7	EAKAKLVPEVKPQPENPEPK	20
	8	KPQPENPEPKEAKAKLVPEV	20
	9	EAKAKLVPEVKPQPENPEPK	20
	10	KPQPENPEPKPQPEGEKPSV	20
	11	PQPEGEKPSVPDINQEKEKA	20
	12	PDINQEKEKAKLAIATYMSK	20
	13	KLAIATYMSKILDDIKKHHL	20
	14	ILDDIKKHHLKKEKHHQIVA	20
	15	KKEKHHQIVALIKDLDKLKK	20
	16	LIKDLDKLKKQALSEIDNVN	20
	17	QALSEIDNVNTKVEIENTVH	20
	18	TKVEIENTVHKVFADMDTVV	20
	19	TVHKVFADMDTVVTKFQKGL	20
	20	PFTIPTKITVGDKVFTVTEV	20
	21	GDKVFTVTEVASQAFSYYPD	20
	22	ASQAFSYYPDETGRIVYYPS	20
	23	ETGRIVYYPSSITIPSSIKK	20
	24	SITIPSSIKKIQKKGFHGSK	20
	25	IQKKGFHGSKAKTIIFDKGS	20
	26	AKTIIFDKGSQLEKIEDRAF	20

	27	QLEKIEDRAFDFSELEEIEL	20
	28	DFSELEEIELPASLEYIGTS	20
	29	PASLEYIGTSFSFSQKLKKL	20
	30	FSFSQKLKKLTFSSSSKLEL	20
	31	TFSSSSKLELISHEAFANLS	20
	32	ISHEAFANLSNLEKLTLPKS	20
	33	EAFANLSNLEKLTLPKSVKT	20
1_2_10 clade 2	34	GNNINSLPSFFLSGVLDSLK	20
	35	FLSGVLDSLKEIHIKNKSTE	20
	36	EIHIKNKSTEFSVKKDTFAI	20
	37	FSVKKDTFAIPETVKFYVTS	20
	38	PETVKFYVTSEHIKDVLKSN	20
	39	EHIKDVLKSNLSTSNDIIVE	20
	40	LSTSNDIIVEKVDNIKQETD	20
	41	KVDNIKQETDVAKPKKNSNQ	20
	42	DVAKPKKNSNQGVVGWVKDK	20
1_2_10 clade 3.2.1	43	AKKEVEAKELEIEKLQDEIS	20
	44	EIEKLQDEISTLEQEVATAQ	20
	45	DEISTLEQEVATAQHQVDNL	20
	46	TELEKLLDSLDPEG	14
Ply	47	AKWHQDYGQVNNVPARMQYE	20
	48	NNVPARMQYEKITAHSMEQL	20
	49	KITAHSMEQLKVKFGSDFEK	20
	50	KVKFGSDFEKTGNSLDIDFN	20
	51	NVRNLSVKIREC	12
PsaA	52	ADLIFYNGINL	11
	53	GAFKYFSKAYG	11
Ply HDC (C01)	54	TSKSDEVEAAFEALIKG	17

PfbA HDC (C06i)	55	DNNSSLSTDNNGGVSINQNK	20
PotD	56	AIILVLWGIATHLDSKINSR	20
	57	LWGIATHLDSKINSRDSQKL	20
	58	FDSNEAMYTKIKQGGTTYDI	20
	59	EAMYTKIKQGGTTYDIAIPS	20
	60	QQLEETVDKLYKLTPNIKA	19
	61	AIGVTFSGEASQMLEKNENL	20
	62	SGEASQMLEKNENLRYVVPT	20
NanA	63	TWHAGEAVNDNRQVDGQKIH	20
	64	NRQVDGQKIHSSTMNNRRAQ	20
	65	SSTMNNRRAQNTESTVVQLN	20
	66	NTESTVVQLNNGDVKLFMRG	20
	67	TESTVVQLNNGDVKLFMRGL	20
	68	PKRENGMVHLARVEENGELT	20
	69	ARVEENGELTWLKHNPIQKG	20
	70	WLKHNPIQKGEFAYNSLQEL	20
	71	EFAYNSLQELGNGEYGILYE	20
	72	GNGEYGILYEHTEKGQNAYT	20
	73	NGEYGILYEHTEKGQNAYTL	20
NanB	74	NYFRIPTLYTFSNGRVFSSI	20
	75	TFSNGRVFSSIDARYGGTHD	20
	76	KINIATSYSDDNGKTW	16
	77	SYGTQLSAIKYSQLIDGKEA	20
	78	IKYSQLIDGKEAVILSTPNS	20
	79	VPMNVFYKDSLFKVTPTNYI	20
	80	LFKVTPTNYIAMTTSQNRGE	20
	81	AMTTSQNRGESWEQFKLLPP	20
	82	MTTSQNRGESWEQFKLLPPF	20
	83	VHMNIFYKDALFKVVPTNYI	20

	84	LFKVVPTNYIAYISSNDHGE	20
	85	AYISSNDHGESWSAPTLLPP	20
	86	YISSNDHGESWSAPTLLPPI	20
PepO	87	SWSKWNQPVDYKEWGMPAHM	20
	88	YKEWGMPAHMVNAYYNPQKN	20
	89	VNAYYNPQKNLIVFPAAILQ	20
	90	LIVFPAAILQAPFYDLHQSS	20
	91	APFYDLHQSSSANYGGIGAV	20
	92	SSSANYGGIGAVIAHEISHA	20
PfbA	93	TGLFTDDGAQVEWGP	15
	94	MNGALNEEGTKAKNLPLINS	20
	95	PLTRKGFPYALNDDGKKS	18
GlpO	96	DLLIIGGGITGAGVALQAAA	20
	97	GAGVALQAAASGLETGLIEM	20
	98	SGLETGLIEMQDFAEGTSSR	20
	99	QDFAEGTSSRSTKLVHGGLR	20
	100	STKLVHGGLRYLKQFDVEVV	20
	101	GLRYLKQFDVEVVSDTVSER	20
	102	DIESSWAGLRPLIA	14
	103	SKEKTREDVESAVSKLESST	20
	104	SAVSKLESSTSEKHLDPSAV	20
	105	SEKHLDPSAVSRGSSLDRDD	20
	106	SRGSSLDRDDNGLLTLAGGK	20
	107	NGLLTLAGGKITDYRKMAEG	20
	108	ITDYRKMAEGAMERVVDILK	20
	109	TDYRKMAEGAMERVVDILKA	20
MalX	110	KTDDTTKSLVTAANGKVYGA	20
	111	TAANGKVYGAPAVIESLVMY	20
	112	PAVIESLVMYYNKDLVKDAP	20
	113	YNKDLVKDAPKTFADLENLA	20

	114	KTFADLENLAKDSKYAFAGE	20
	115	KDSKYAFAGEDGKTTAFLAD	20
	116	DGKTTAFLADWTNFYYTYGL	20
	117	GKTTAFLADWTNFYYTYGLL	20
	118	FQEGKTAAIIDGPWKAQAFK	20
	119	DGPWKAQAFKDAKVNYGVAT	20
	120	DAKVNYGVATIPTLPNGKEY	20
	121	GVATIPTLPNGKEYAAFGGG	20
	122	IPANTEARSYAEGKNDELTT	20
	123	AEGKNDELTTAVIKQFKNTQ	20
	124	AVIKQFKNTQPLPNISQMSA	20
	125	PLPNISQMSAVWDPAKNMLF	20
	126	TSKSDEVEAAFEALIKG	17
IgA1 HDC (C02)	127	YDRYRSSEYPKGEKLNKFVE	20
	128	KGEKLNKFVEENAHEAAKRF	20
	129	ENAHEAAKRFRNHYDYWYKI	20
	130	RNHYDYWYKILDNDNKEKLF	20
	131	LDNDNKEKLFRSVLVYDAFR	20
	132	RSVLVYDAFRFGNDKNDKIQ	20
	133	VLVYDAFRFGNDKNDKIQEA	20
IgA1 HDC (C03)	134	NMFDLIYTLEILEGRAVAKL	20
	135	ILEGRAVAKLGYNEKNDLLR	20
	136	GYNEKNDLLRKIENIYKKDP	20
	137	KIENIYKKDPDGNQVYATNA	20
	138	KKDPDGNQVYATNAIRRLTP	20
ZmpB HDC (C04)	139	QLPTFVKTMFENEWLHINGE	20
	140	ENEWLHINGESSGAVAALRQ	20

	141	SSGAVAALRQKIMDNKTAIL	20
	142	KIMDNKTAILLALTYINRYY	20
	143	NKTAILLALTYINRYYDVKF	20
ZmpB HDC (C05)	144	WYAYHGYGAHAGGRNRVNYE	20
	145	AGGRNRVNYEVFDVLSEYGI	20
	146	VFDVLSEYGISVFTHELTHV	20
	147	SVFTHELTHVNDTWIYLGGY	20
	148	FTHELTHVNDTWIYLGGYGR	20
PfbA HDC (C06 ii - iv)	149	NGGVSINQNKPDITNDTMMG	20
	150	PDITNDTMMGNISGMEVNTI	20
	151	TMMGNISGMEVNTILSTNLN	20

C – control (followed by identification number); HDC – highly diverse control

Appendix B Epitope lymphoproliferative response rankings

	Volunteer 1				Volunteer 2			
Ranking	Epitope	PF	Epitope	UGPI	Epitope	PF	Epitope	UGPI
1	32	0.46	1	18.92	25	0.33	22	6.63
2	50	0.44	38	17.07	24	0.29	21	6.57
3	2	0.43	28	15.54	23	0.28	23	6.41
4	54	0.42	36	15.07	21	0.25	20	6.35
5	44	0.41	30	15.06	3	0.24	24	5.50
6	46	0.41	29	14.53	16	0.24	19	5.43
7	42	0.41	4	13.92	20	0.22	18	4.96
8	45	0.41	3	13.70	22	0.21	25	4.90
9	1	0.41	45	13.67	19	0.21	17	3.64
10	52	0.40	47	13.57	15	0.21	16	3.62
11	51	0.40	26	13.57	17	0.19	15	2.11
12	48	0.40	50	13.48	18	0.19	9	1.66
13	43	0.39	35	13.46	14	0.17	14	1.53
14	31	0.38	44	13.28	13	0.17	11	1.42
15	55	0.38	2	13.15	12	0.14	10	0.90
16	53	0.38	37	12.89	11	0.14	12	0.89
17	38	0.37	54	12.59	9	0.12	13	0.85
18	37	0.37	41	12.43	8	0.11	30	0.20
19	4	0.37	52	12.41	10	0.09	29	0.08
20	3	0.35	49	12.30	7	0.07	4	0.03
21	40	0.35	27	12.23	50	0.03	36	-0.01
22	47	0.33	34	12.05	32	0.02	47	-0.09
23	39	0.33	42	12.03	36	0.01	2	-0.10
24	49	0.32	40	12.00	2	0.01	39	-0.11
25	36	0.32	33	11.92	52	0.01	28	-0.12
26	41	0.31	48	11.88	44	0.01	54	-0.14

27	34	0.30	43	11.48	4	0.01	41	-0.15
28	35	0.28	32	11.42	1	0.00	49	-0.16
29	26	0.26	53	11.37	46	0.00	26	-0.17
30	33	0.26	39	11.36	33	0.00	7	-0.18
31	29	0.20	55	11.20	6	0.00	43	-0.19
32	28	0.09	51	11.03	5	-0.02	45	-0.20
33	27	0.09	46	10.81	45	-0.02	51	-0.20
34	30	0.06	31	10.49	34	-0.02	42	-0.21
35	13	-0.03	25	2.33	38	-0.02	35	-0.23
36	16	-0.03	21	1.90	39	-0.03	37	-0.23
37	24	-0.03	20	1.61	40	-0.04	55	-0.23
38	12	-0.04	22	1.35	51	-0.04	48	-0.25
39	17	-0.04	23	1.15	42	-0.06	27	-0.27
40	23	-0.04	17	1.04	43	-0.07	1	-0.30
41	22	-0.05	24	1.02	41	-0.07	38	-0.33
42	15	-0.05	19	0.82	54	-0.07	31	-0.34
43	21	-0.05	5	0.66	37	-0.08	34	-0.34
44	25	-0.05	16	0.63	55	-0.08	44	-0.34
45	19	-0.06	18	0.23	53	-0.09	53	-0.39
46	10	-0.06	15	0.21	27	-0.09	32	-0.41
47	20	-0.06	9	0.08	35	-0.09	50	-0.42
48	7	-0.06	13	-0.04	49	-0.10	46	-0.47
49	11	-0.06	14	-0.07	48	-0.10	33	-0.49
50	18	-0.07	8	-0.14	47	-0.10	8	-0.50
51	14	-0.07	7	-0.43	31	-0.12	40	-0.56
52	6	-0.07	12	-0.46	28	-0.12	5	-0.57
53	8	-0.08	11	-0.62	26	-0.13	52	-0.62
54	5	-0.19	10	-0.68	29	-0.13	3	-1.02
55	9	-0.20	6	-0.71	30	-0.23	6	-1.28

-	US	0.66	US	12.58	US	0.37	US	6.95
-	PHA-L	-0.08	PHA-L	60.47	PHA-L	0.29	PHA-L	0.29

Top twenty epitopes coloured per volunteer: volunteer 1 (green) and volunteer 2 (blue). PF = precursor frequency; UGPI = upper generation proliferation index; PHA-L = leucoagglutinating phytohemagglutinin.

References

ABBOTT, D. W., HIGGINS, M. A., HYRNUIK, S., PLUVINAGE, B., VAN BUEREN, A. L. & BORASTON, A. B. 2010. The molecular basis of glycogen breakdown and transport in *Streptococcus pneumoniae*. *Molecular Microbiology,* 77**,** 183-199.

AGARWAL, V., KUCHIPUDI, A., FULDE, M., RIESBECK, K., BERGMANN, S. & BLOM, A. M. 2013. *Streptococcus pneumoniae* Endopeptidase O (PepO) Is a Multifunctional Plasminogen- and Fibronectin-binding Protein, Facilitating Evasion of Innate Immunity and Invasion of Host Cells. *Journal of Biological Chemistry,* 288**,** 6849-6863.

AGGARWAL, R., SHUKLA, R., JAMEEL, S., AGRAWAL, S., PURI, P., GUPTA, V. K., PATIL, A. P. & NAIK, S. 2007. T-cell epitope mapping of ORF2 and ORF3 proteins of human hepatitis E virus. *J Viral Hepat,* 14**,** 283-92.

AHMED, M. S., DERBYSHIRE, S., FLANAGAN, B., LOH, C., MCCORMICK, M., BAROCCHI, M., MASIGNANI, V., FINN, A. & ZHANG, Q. B. 2014. Immune responses to pneumococcal pilus RrgA and RrgB antigens and their relationship with pneumococcal carriage in humans. *Journal of Infection,* 68**,** 562-571.

AL-SWAILEM, A. M., KADRY, A. A., FOUDA, S. I., SHIBL, A. M. & SHAIR, O. H. 2004. Phenotypic and genotypic characterization of invasive *Streptococcus pneumoniae* clinical isolates. *Current Therapeutic Research-Clinical and Experimental,* 65**,** 423-432.

ALBIGER, B., DAHLBERG, S., SANDGREN, A., WARTHA, F., BEITER, K., KATSURAGI, H., AKIRA, S., NORMARK, S. & HENRIQUES-NORMARK, B. 2007. Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. *Cellular Microbiology,* 9**,** 633-644.

ALI, Y. M., LYNCH, N. J., HALEEM, K. S., FUJITA, T., ENDO, Y., HANSEN, S., HOLMSKOV, U., TAKAHASHI, K., STAHL, G. L., DUDLER, T., GIRIJA, U. V., WALLIS, R., KADIOGLU, A., STOVER, C. M., ANDREW, P. W. & SCHWAEBLE, W. J. 2012. The Lectin Pathway of Complement Activation Is a Critical Component of the Innate Immune Response to Pneumococcal Infection. *Plos Pathogens,* 8.

ALMEIDA, S. T., NUNES, S., SANTOS PAULO, A. C., VALADARES, I., MARTINS, S., BREIA, F., BRITO-AVO, A., MORAIS, A., DE LENCASTRE, H. & SA-LEAO, R. 2014. Low Prevalence of Pneumococcal Carriage and High Serotype and Genotype Diversity among Adults over 60 Years of Age Living in Portugal. *Plos One,* 9.

APPELBAUM, P. C. 1992. Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. *Clin Infect Dis,* 15**,** 77-83.

ATTALI, C., FROLET, C., DURMORT, C., OFFANT, J., VERNET, T. & DI GUILMI, A. M. 2008. *Streptococcus pneumoniae* choline-binding protein E interaction with plasminogen/plasmin stimulates migration across the extracellular matrix. *Infection and Immunity,* 76**,** 466-476.

AUSTRIAN, R., DOUGLAS, R. M., SCHIFFMAN, G., COETZEE, A. M., KOORNHOF, H. J., HAYDEN-SMITH, S. & REID, R. D. 1976. Prevention of pneumococcal pneumonia by vaccination. *Trans Assoc Am Physicians,* 89**,** 184-94.

BACCHETTA, R., GAMBINERI, E. & RONCAROLO, M. G. 2007. Role of regulatory T cells and FOXP3 in human diseases. *J Allergy Clin Immunol,* 120**,** 227-35; quiz 236-7.

BAGNOLI, F., MOSCHIONI, M., DONATI, C., DIMITROVSKA, V., FERLENGHI, I., FACCIOTTI, C., MUZZI, A., GIUSTI, F., EMOLO, C., SINISI, A., HILLERINGMANN, M., PANSEGRAU, W., CENSINI, S., RAPPUOLI, R., COVACCI, A., MASIGNANI, V. & BAROCCHI, M. A. 2008. A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. *Journal of Bacteriology,* 190**,** 5480-5492.

BAJAJ, M., MAMIDYALA, S. K., ZUEGG, J., BEGG, S. L., WEEN, M. P., LUO, Z. Y., HUANG, J. X., MCEWAN, A. G., KOBE, B., PATON, J. C., MCDEVITT, C. A. & COOPER, M. A. 2015. Discovery of Novel Pneumococcal Surface Antigen A (PsaA) Inhibitors Using a Fragment-based Drug Design Approach. *Acs Chemical Biology,* 10**,** 1511-1520.

BALABAN, M., BATTIG, P., MUSCHIOL, S., TIRIER, S. M., WARTHA, F., NORMARK, S. & HENRIQUES-NORMARK, B. 2014. Secretion of a pneumococcal type II secretion system pilus correlates with DNA uptake during transformation. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** E758-E765.

BALACHANDRAN, P., BROOKS-WALTER, A., VIROLAINEN-JULKUNEN, A., HOLLINGSHEAD, S. K. & BRILES, D. E. 2002. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. *Infection and Immunity,* 70**,** 2526-2534.

BALHARA, J. & GOUNNI, A. S. 2012. The alveolar macrophages in asthma: a double-edged sword. *Mucosal Immunology,* 5**,** 605-609.

BANGERT, M., WRIGHT, A. K., RYLANCE, J., KELLY, M. J., WRIGHT, A. D., CARLONE, G. M., SAMPSON, J. S., RAJAM, G., ADES, E. W., KADIOGLU, A. & GORDON, S. B. 2013. Immunoactivating peptide P4 augments alveolar macrophage phagocytosis in two diverse human populations. *Antimicrob Agents Chemother,* 57**,** 4566-9.

BARTRAM, U. & SPEER, C. P. 2004. The role of transforming growth factor beta in lung development and disease. *Chest,* 125**,** 754-65.

BASSET, A., THOMPSON, C. M., HOLLINGSHEAD, S. K., BRILES, D. E., ADES, E. W., LIPSITCH, M. & MALLEY, R. 2007. Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. *Infect Immun,* 75**,** 5460-4.

BATTEN, M. R., SENIOR, B. W., KILIAN, M. & WOOF, J. M. 2003. Amino acid sequence requirements in the hinge of human immunoglobulin A1 (IgA1) for cleavage by streptococcal IgA1 proteases. *Infection and Immunity,* 71**,** 1462-1469.

BEITER, K., WARTHA, F., ALBIGER, B., NORMARK, S., ZYCHLINSKY, A. & HENRIQUES-NORMARK, B. 2006. An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. *Current Biology,* 16**,** 401-407.

BERRY, A. M. & PATON, J. C. 1996. Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*. *Infection and Immunity,* 64**,** 5255-5262.

BEWLEY, M. A., PHAM, T. K., MARRIOTT, H. M., NOIREL, J., CHU, H. P., OW, S. Y., RYAZANOV, A. G., READ, R. C., WHYTE, M. K., CHAIN, B., WRIGHT, P. C. & DOCKRELL, D. H. 2011. Proteomic evaluation and validation of cathepsin D regulated proteins in macrophages exposed to *Streptococcus pneumoniae*. *Mol Cell Proteomics,* 10**,** M111.008193.

BLACK, S., SHINEFIELD, H., FIREMAN, B., LEWIS, E., RAY, P., HANSEN, J. R., ELVIN, L., ENSOR, K. M., HACKELL, J., SIBER, G., MALINOSKI, F., MADORE, D., CHANG, I., KOHBERGER, R., WATSON, W., AUSTRIAN, R. & EDWARDS, K. 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J,* 19**,** 187-95.

BLUE, C. E., PATERSON, G. K., KERR, A. R., BERGE, M., CLAVERYS, J. P. & MITCHELL, T. J. 2003. ZmpB, a novel virulence factor of *Streptococcus pneumoniae* that induces tumor necrosis factor alpha production in the respiratory tract. *Infection and Immunity,* 71**,** 4925-4935.

BOGAERT, D., HERMANS, P. W. M., ADRIAN, P. V., RUMKE, H. C. & DE GROOT, R. 2004. Pneumococcal vaccines: an update on current strategies. *Vaccine,* 22**,** 2209-2220.

BOGAERT, D., WEINBERGER, D., THOMPSON, C., LIPSITCH, M. & MALLEY, R. 2009. Impaired Innate and Adaptive Immunity to *Streptococcus pneumoniae* and Its Effect on Colonization in an Infant Mouse Model. *Infection and Immunity,* 77**,** 1613-1622.

BONTEN, M. J. M., HUIJTS, S. M., BOLKENBAAS, M., WEBBER, C., PATTERSON, S., GAULT, S., VAN WERKHOVEN, C. H., VAN DEURSEN, A. M. M., SANDERS, E. A. M., VERHEIJ, T. J. M., PATTON, M., MCDONOUGH, A., MORADOGHLI-HAFTVANI, A., SMITH, H., MELLELIEU, T., PRIDE, M. W., CROWTHER, G., SCHMOELE-THOMA, B., SCOTT, D. A., JANSEN, K. U., LOBATTO, R., OOSTERMAN, B., VISSER, N., CASPERS, E., SMORENBURG, A., EMINI, E. A., GRUBER, W. C. & GROBBEE, D. E. 2015. Polysaccharide Conjugate Vaccine against Pneumococcal Pneumonia in Adults. *New England Journal of Medicine,* 372**,** 1114-1125.

BOSARGE, J. R., ETHRIDGE, A., MOORE, Q. & MCDANIEL, L. S. 2001. DNA vaccination with regions within the alpha-helical domain of PspA/Rx1 protects against *Streptococcus pneumoniae*. *Abstracts of the General Meeting of the American Society for Microbiology,* 101**,** 338-338.

BRANGER, J., KNAPP, S., WEIJER, S., LEEMANS, J. C., PATER, J. M., SPEELMAN, P., FLORQUIN, S. R. & VAN DER POLL, T. 2004. Role of toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. *Infection and Immunity,* 72**,** 788-794.

BRIDY-PAPPAS, A. E., MARGOLIS, M. B., CENTER, K. J. & ISAACMAN, D. J. 2005. *Streptococcus pneumoniae*: description of the pathogen, disease epidemiology, treatment, and prevention. *Pharmacotherapy,* 25**,** 1193-212.

BRILES, D. E., HOLLINGSHEAD, S. K., KING, J., SWIFT, A., BRAUN, P. A., PARK, M. K., FERGUSON, L. M., NAHM, M. H. & NABORS, G. S. 2000a. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. *Journal of Infectious Diseases,* 182**,** 1694-1701.

BRILES, D. E., HOLLINGSHEAD, S. K., NABORS, G. S., PATON, J. C. & BROOKS-WALTER, A. 2000b. The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*. *Vaccine,* 19**,** S87-S95.

BRINKMANN, V., REICHARD, U., GOOSMANN, C., FAULER, B., UHLEMANN, Y., WEISS, D. S., WEINRAUCH, Y. & ZYCHLINSKY, A. 2004. Neutrophil extracellular traps kill bacteria. *Science,* 303**,** 1532-5.

BRITTAN, J. L., BUCKERIDGE, T. J., FINN, A., KADIOGLU, A. & JENKINSON, H. F. 2012. Pneumococcal neuraminidase A: an essential upper airway colonization factor for *Streptococcus pneumoniae*. *Molecular Oral Microbiology,* 27**,** 270-283.

BROOKS-WALTER, A., BRILES, D. E. & HOLLINGSHEAD, S. K. 1999. The pspC gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. *Infection and Immunity,* 67**,** 6533-6542.

BROWN, J. S., GILLILAND, S. M. & HOLDEN, D. W. 2001. A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Molecular Microbiology,* 40**,** 572-585.

BROWN, J. S., HUSSELL, T., GILLILAND, S. M., HOLDEN, D. W., PATON, J. C., EHRENSTEIN, M. R., WALPORT, M. J. & BOTTO, M. 2002. The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 16969-16974.

BUCKLEY, R. H. 2004. Pulmonary complications of primary immunodeficiencies. *Paediatr Respir Rev,* 5 Suppl A**,** S225-33.

BURNHAM, E. L., PHANG, T. L., HOUSE, R., VANDIVIER, R. W., MOSS, M. & GAYDOS, J. 2011. Alveolar macrophage gene expression is altered in the setting of alcohol use disorders. *Alcohol Clin Exp Res,* 35**,** 284-94.

CARVALHO, M. D. G. S., TONDELLA, M. L., MCCAUSTLAND, K., WEIDLICH, L., MCGEE, L., MAYER, L. W., STEIGERWALT, A., WHALEY, M., FACKLAM, R. R., FIELDS, B., CARLONE, G., ADES, E. W., DAGAN, R. & SAMPSON, J. S. 2007. Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. *Journal of Clinical Microbiology,* 45**,** 2460-2466.

CHAI, M. H., WEILAND, F., HARVEY, R. M., HOFFMANN, P., OGUNNIYI, A. D. & PATON, J. C. 2017. Proteomic comparisons of opaque and transparent variants of *Streptococcus pneumoniae* by two dimensional-differential gel electrophoresis. *Sci Rep,* 7**,** 2453.

CHALMERS, J. D., CAMPLING, J., DICKER, A., WOODHEAD, M. & MADHAVA, H. 2016. A systematic review of the burden of vaccine preventable pneumococcal disease in UK adults. *Bmc Pulmonary Medicine,* 16.

CHANDLER, R. L. 1970. ULTRASTRUCTURAL PATHOLOGY OF MASTITIS IN MOUSE - STUDY OF EXPERIMENTAL STAPHYLOCOCCAL AND STREPTOCOCCAL INFECTIONS. *British Journal of Experimental Pathology,* 51**,** 639-&.

CHANDRA, H. & YADAV, J. S. 2016. T-cell antigens of Mycobacterium immunogenum, an etiological agent of occupational hypersensitivity pneumonitis. *Mol Immunol,* 75**,** 168-77.

CHENG, Q., FINKEL, D. & HOSTETTER, M. K. 2000. Novel purification scheme and functions for a C3-binding protein from *Streptococcus pneumoniae*. *Biochemistry,* 39**,** 5450-5457.

CHODOSH, S. 1987. ACUTE BACTERIAL EXACERBATIONS IN BRONCHITIS AND ASTHMA. *American Journal of Medicine,* 82**,** 154-163.

CLUTTERBUCK, E. A., LAZARUS, R., YU, L.-M., BOWMAN, J., BATEMAN, E. A. L., DIGGLE, L., ANGUS, B., PETO, T. E., BEVERLEY, P. C., MANT, D. & POLLARD, A. J. 2012.

Pneumococcal Conjugate and Plain Polysaccharide Vaccines Have Divergent Effects on Antigen-Specific B Cells. *Journal of Infectious Diseases,* 205**,** 1408-1416.
COBEY, S. & LIPSITCH, M. 2012. Niche and neutral effects of acquired immunity permit coexistence of pneumococcal serotypes. *Science,* 335**,** 1376-80.
COHEN, J. M., KHANDAVILLI, S., CAMBERLEIN, E., HYAMS, C., BAXENDALE, H. E. & BROWN, J. S. 2011. Protective Contributions against Invasive *Streptococcus pneumoniae* Pneumonia of Antibody and Th17-Cell Responses to Nasopharyngeal Colonisation. *Plos One,* 6.
COHEN, J. M., WILSON, R., SHAH, P., BAXENDALE, H. E. & BROWN, J. S. 2013. Lack of cross-protection against invasive pneumonia caused by heterologous strains following murine *Streptococcus pneumoniae* nasopharyngeal colonisation despite whole cell ELISAs showing significant cross-reactive IgG. *Vaccine,* 31**,** 2328-32.
COLE, T. S. & CANT, A. J. 2010. Clinical experience in T cell deficient patients. *Allergy, Asthma & Clinical Immunology,* 6**,** 9.
COLLINS, A. M., JOHNSTONE, C. M. K., GRITZFELD, J. F., BANYARD, A., HANCOCK, C. A., WRIGHT, A. D., MACFARLANE, L., FERREIRA, D. M. & GORDON, S. B. 2016. Pneumococcal Colonization Rates in Patients Admitted to a United Kingdom Hospital with Lower Respiratory Tract Infection: a Prospective Case-Control Study. *Journal of Clinical Microbiology,* 54**,** 944-949.
COLLINS, A. M., RYLANCE, J., WOOTTON, D. G., WRIGHT, A. D., WRIGHT, A. K., FULLERTON, D. G. & GORDON, S. B. 2014. Bronchoalveolar lavage (BAL) for research; obtaining adequate sample yield. *J Vis Exp*.
COLLINS, A. M., WRIGHT, A. D., MITSI, E., GRITZFELD, J. F., HANCOCK, C. A., PENNINGTON, S. H., WANG, D., MORTON, B., FERREIRA, D. M. & GORDON, S. B. 2015. First human challenge testing of a pneumococcal vaccine. Double-blind randomized controlled trial. *Am J Respir Crit Care Med,* 192**,** 853-8.
CRAIN, M. J., WALTMAN, W. D., TURNER, J. S., YOTHER, J., TALKINGTON, D. F., MCDANIEL, L. S., GRAY, B. M. & BRILES, D. E. 1990. Pneumococcal surface protein-A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of *Streptococcus pneumoniae*. *Infection and Immunity,* 58**,** 3293-3299.
CREMERS, A. J. H., ZOMER, A. L., GRITZFELD, J. F., FERWERDA, G., VAN HIJUM, S. A. F. T., FERREIRA, D. M., SHAK, J. R., KLUGMAN, K. P., BOEKHORST, J., TIMMERMAN, H. M., DE JONGE, M. I., GORDON, S. B. & HERMANS, P. W. M. 2014. The adult nasopharyngeal microbiome as a determinant of pneumococcal acquisition. *Microbiome,* 2.
CRUMPTON, M. J., ALLAN, D., AUGER, J., GREEN, N. M. & MAINO, V. C. 1975. Recognition at cell surfaces: phytohaemagglutinin-lymphocyte interaction. *Philos Trans R Soc Lond B Biol Sci,* 272**,** 173-80.
CUA, D. J. & TATO, C. M. 2010. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol,* 10**,** 479-89.
CURTISS, R., XIN, W., LI, Y. H., KONG, W., WANDA, S. Y., GUNN, B. & WANG, S. F. 2010. New Technologies in Using Recombinant Attenuated Salmonella Vaccine Vectors. *Critical Reviews in Immunology,* 30**,** 255-270.
CUTTS, F. T., ZAMAN, S. M. A., ENWERE, G., JAFFAR, S., LEVINE, O. S., OKOKO, J. B., OLUWALANA, C., VAUGHAN, A., OBARO, S. K., LEACH, A., MCADAM, K. P., BINEY, E., SAAKA, M., ONWUCHEKWA, U., YALLOP, F., PIERCE, N. F., GREENWOOD, B. M., ADEGBOLA, R. A. & GAMBIAN PNEUMOCOCCAL VACCINE, T. 2005. Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. *Lancet,* 365**,** 1139-1146.
DAGAN, R. & FRASER, D. 2000. Conjugate pneumococcal vaccine and antibiotic-resistant *Streptococcus pneumoniae*: herd immunity and reduction of otitis morbidity. *Pediatric Infectious Disease Journal,* 19**,** S79-S88.
DANIELS, C. C., COAN, P., KING, J., HALE, J., BENTON, K. A., BRILES, D. E. & HOLLINGSHEAD, S. K. 2010. The Proline-Rich Region of Pneumococcal Surface Proteins A and C Contains Surface-Accessible Epitopes Common to All Pneumococci and Elicits Antibody-Mediated Protection against Sepsis. *Infection and Immunity,* 78**,** 2163-2172.

DARKES, M. J. & PLOSKER, G. L. 2002. Pneumococcal conjugate vaccine (Prevnar; PNCRM7): a review of its use in the prevention of *Streptococcus pneumoniae* infection. *Paediatr Drugs,* 4**,** 609-30.

DARRIEUX, M., MIYAJI, E. N., FERREIRA, D. M., LOPES, L. M., LOPES, A. P. Y., REN, B., BRILES, D. E., HOLLINGSHEAD, S. K. & LEITEL, L. C. C. 2007. Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against *Streptococcus pneumoniae* that correlates with antibody-mediated enhancement of complement deposition. *Infection and Immunity,* 75**,** 5930-5938.

DARRIEUX, M., MORENO, A. T., FERREIRA, D. M., PIMENTA, F. C., DE ANDRADE, A., LOPES, A. P. Y., LEITE, L. C. C. & MIYAJI, E. N. 2008. Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. *Journal of Medical Microbiology,* 57**,** 273-278.

DAVE, S., BROOKS-WALTER, A., PANGBURN, M. K. & MCDANIEL, L. S. 2001. PspC, a pneumococcal surface protein, binds human factor H. *Infection and Immunity,* 69**,** 3435-3437.

DAVIS, K. M., AKINBI, H. T., STANDISH, A. J. & WEISER, J. N. 2008. Resistance to Mucosal Lysozyme Compensates for the Fitness Deficit of Peptidoglycan Modifications by *Streptococcus pneumoniae*. *Plos Pathogens,* 4.

DICKSON, R. P. & HUFFNAGLE, G. B. 2015. The Lung Microbiome: New Principles for Respiratory Bacteriology in Health and Disease. *PLoS Pathog,* 11**,** e1004923.

DIETHELM-OKITA, B. M., RAJU, R., OKITA, D. K. & CONTI-FINE, B. M. 1997. Epitope repertoire of human CD4+ T cells on tetanus toxin: identification of immunodominant sequence segments. *J Infect Dis,* 175**,** 382-91.

DITSE, Z., ADRIAN, P. V., KUWANDA, L. & MADHI, S. A. 2013. Association of *Streptococcus pneumoniae* common protein antigen (CPA) antibodies and pneumococcal nasopharyngeal colonization in HIV-infected and HIV-uninfected African children. *Vaccine,* 31**,** 4421-7.

DOCKRELL, D. H., MARRIOTT, H. M., PRINCE, L. R., RIDGER, V. C., INCE, P. G., HELLEWELL, P. G. & WHYTE, M. K. 2003. Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. *J Immunol,* 171**,** 5380-8.

DONG, J., WANG, J., HE, Y. J., LI, C. W., ZHOU, A., CUI, J., XU, W. C., ZHONG, L., YIN, Y. B., ZHANG, X. M. & WANG, H. 2014. GHIP in *Streptococcus pneumoniae* is involved in antibacterial resistance and elicits a strong innate immune response through TLR2 and JNK/p38MAPK. *Febs Journal,* 281**,** 3803-3815.

DOUGLAS, R. M., PATON, J. C., DUNCAN, S. J. & HANSMAN, D. J. 1983. Antibody response to pneumococcal vaccination in children younger than 5-years of age. *Journal of Infectious Diseases,* 148**,** 131-137.

DOWSON, C. G. 2004. What Is a Pneumococcus?

ESKOLA, J., KILPI, T., PALMU, A., JOKINEN, J., HAAPAKOSKI, J., HERVA, E., TAKALA, A., KAYHTY, H., KARMA, P., KOHBERGER, R., SIBER, G., MAKELA, P. H., LOCKHART, S., ECROLA, M. & FINNISH OTITIS MEDIA STUDY, G. 2001. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *New England Journal of Medicine,* 344**,** 403-409.

FAN, R., XIANG, Y., YANG, L., LIU, Y., CHEN, P., WANG, L., FENG, W., YIN, K., FU, M., XU, Y. & WU, J. 2016. Impaired NK cells' activity and increased numbers of CD4+ CD25+ regulatory T cells in multidrug-resistant Mycobacterium tuberculosis patients. *Tuberculosis (Edinb),* 98**,** 13-20.

FEDSON, D. S. 1999. The clinical effectiveness of pneumococcal vaccination: a brief review. *Vaccine,* 17**,** S85-S90.

FERREIRA 2013. Controlled Human Infection and Rechallenge with *Streptococcus pneumoniae* Reveals the Protective Efficacy of Carriage in Healthy Adults (vol 187, pg 855, 2013). *American Journal of Respiratory and Critical Care Medicine,* 187**,** 1153-1153.

FERREIRA, D. M., NEILL, D. R., BANGERT, M., GRITZFELD, J. F., GREEN, N., WRIGHT, A. K. A., PENNINGTON, S. H., MORENO, L. B., MORENO, A. T., MIYAJI, E. N., WRIGHT, A. D., COLLINS, A. M., GOLDBLATT, D., KADIOGLU, A. & GORDON, S. B. 2013. Controlled Human Infection and Rechallenge with *Streptococcus pneumoniae* Reveals the Protective Efficacy of Carriage in Healthy Adults. *American Journal of Respiratory and Critical Care Medicine,* 187**,** 855-864.

FITCHES, E., ILETT, C., GATEHOUSE, A. M. R., GATEHOUSE, L. N., GREENE, R., EDWARDS, J. P. & GATEHOUSE, J. A. 2001. The effects of Phaseolus vulgaris erythro- and leucoagglutinating isolectins (PHA-E and PHA-L) delivered via artificial diet and transgenic plants on the growth and development of tomato moth (Lacanobia oleracea) larvae; lectin binding to gut glycoproteins in vitro and in vivo. *Journal of Insect Physiology,* 47**,** 1389-1398.

FLEURIE, A., CLUZEL, C., GUIRAL, S., FRETON, C., GALISSON, F., ZANELLA-CLEON, I., DI GUILMI, A. M. & GRANGEASSE, C. 2012. Mutational dissection of the S/T-kinase StkP reveals crucial roles in cell division of *Streptococcus pneumoniae*. *Molecular Microbiology,* 83**,** 746-758.

FONTENOT, J. D., GAVIN, M. A. & RUDENSKY, A. Y. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol,* 4**,** 330-6.

GAMEZ, G. & HAMMERSCHMIDT, S. 2012. Combat Pneumococcal Infections: Adhesins as Candidates for Protein-Based Vaccine Development. *Current Drug Targets,* 13**,** 323-337.

GERLINI, A., COLOMBA, L., FURI, L., BRACCINI, T., MANSO, A. S., PAMMOLLI, A., WANG, B., VIVI, A., TASSINI, M., VAN ROOIJEN, N., POZZI, G., RICCI, S., ANDREW, P. W., KOEDEL, U., MOXON, E. R. & OGGIONI, M. R. 2014. The role of host and microbial factors in the pathogenesis of pneumococcal bacteraemia arising from a single bacterial cell bottleneck. *PLoS Pathog,* 10**,** e1004026.

GINSBURG, A. S., NAHM, M. H., KHAMBATY, F. M. & ALDERSON, M. R. 2012. Issues and challenges in the development of pneumococcal protein vaccines. *Expert Review of Vaccines,* 11**,** 279-285.

GLADSTONE, R. A., JEFFERIES, J. M., TOCHEVA, A. S., BEARD, K. R., GARLEY, D., CHONG, W. W., BENTLEY, S. D., FAUST, S. N. & CLARKE, S. C. 2015. Five winters of pneumococcal serotype replacement in UK carriage following PCV introduction. *Vaccine,* 33**,** 2015-2021.

GLENNIE, S., GRITZFELD, J. F., PENNINGTON, S. H., GARNER-JONES, M., COOMBES, N., HOPKINS, M. J., VADESILHO, C. F., MIYAJI, E. N., WANG, D., WRIGHT, A. D., COLLINS, A. M., GORDON, S. B. & FERREIRA, D. M. 2016. Modulation of nasopharyngeal innate defenses by viral coinfection predisposes individuals to experimental pneumococcal carriage. *Mucosal Immunology,* 9**,** 56-67.

GLENNIE, S. J., SEPAKO, E., MZINZA, D., HARAWA, V., MILES, D. J., JAMBO, K. C., GORDON, S. B., WILLIAMS, N. A. & HEYDERMAN, R. S. 2011. Impaired CD4 T cell memory response to *Streptococcus pneumoniae* precedes CD4 T cell depletion in HIV-infected Malawian adults. *PLoS One,* 6**,** e25610.

GLOVER, D. T., HOLLINGSHEAD, S. K. & BRILES, D. E. 2008. *Streptococcus pneumoniae* surface protein PcpA elicits protection against lung infection and fatal sepsis. *Infection and Immunity,* 76**,** 2767-2776.

GORDON, S. & TAYLOR, P. R. 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol,* 5**,** 953-64.

GORDON, S. B., MOLYNEUX, M. E., BOEREE, M. J., KANYANDA, S., CHAPONDA, M., SQUIRE, S. B. & READ, R. C. 2001. Opsonic phagocytosis of *Streptococcus pneumoniae* by alveolar macrophages is not impaired in human immunodeficiency virus-infected Malawian adults. *J Infect Dis,* 184**,** 1345-9.

GRAY, D. & MATZINGER, P. 1991. T cell memory is short-lived in the absence of antigen. *J Exp Med,* 174**,** 969-74.

GREENBERG, D., GIVON-LAVI, N., BROIDES, A., BLANCOVICH, I., PELED, N. & DAGAN, R. 2006. The contribution of smoking and exposure to tobacco smoke to *Streptococcus pneumoniae* and Haemophilus influenzae carriage in children and their mothers. *Clinical Infectious Diseases,* 42**,** 897-903.

GRITZFELD, J. F., WRIGHT, A. D., COLLINS, A. M., PENNINGTON, S. H., WRIGHT, A. K. A., KADIOGLU, A., FERREIRA, D. M. & GORDON, S. B. 2013. Experimental human pneumococcal carriage. *Journal of visualized experiments : JoVE*.

GUY, R., GEOGHEGAN, L., HEGINBOTHOM, M., HOWE, R., MULLER-PEBODY, B., REILLY, J. S., WILSON, J., WIUFF, C., WYATT, T. & JOHNSON, A. P. 2016. Non-susceptibility of Escherichia coli, Klebsiella spp., Pseudomonas spp., *Streptococcus pneumoniae* and Staphylococcus aureus in the UK: temporal trends in England, Northern Ireland, Scotland and Wales. *J Antimicrob Chemother,* 71**,** 1564-9.

HALES, B. J., CHAI, L. Y., ELLIOT, C. E., PEARCE, L. J., ZHANG, G., HEINRICH, T. K., SMITH, W. A., KUSEL, M. M., HOLT, P. G., SLY, P. D. & THOMAS, W. R. 2012. Antibacterial antibody responses associated with the development of asthma in house dust mite-sensitised and non-sensitised children. *Thorax,* 67**,** 321-7.

HAMMERSCHMIDT, S., TALAY, S. R., BRANDTZAEG, P. & CHHATWAL, G. S. 1997. SpsA, a novel pneumococcal surface protein with specific binding to secretory Immunoglobulin A and secretory component. *Molecular Microbiology,* 25**,** 1113-1124.

HEMMI, H., TAKEUCHI, O., KAWAI, T., KAISHO, T., SATO, S., SANJO, H., MATSUMOTO, M., HOSHINO, K., WAGNER, H., TAKEDA, K. & AKIRA, S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature,* 408**,** 740-5.

HERNANI, M. D. L., DUARTE FERREIRA, P. C., FERREIRA, D. M., MIYAJI, E. N., HO, P. L. & SARNO OLIVEIRA, M. L. 2011. Nasal immunization of mice with Lactobacillus casei expressing the pneumococcal surface protein C primes the immune system and decreases pneumococcal nasopharyngeal colonization in mice. *Fems Immunology and Medical Microbiology,* 62**,** 263-272.

HOE, E., BOELSEN, L. K., TOH, Z. Q., SUN, G. W., KOO, G. C., BALLOCH, A., MARIMLA, R., DUNNE, E. M., TIKODUADUA, L., RUSSELL, F. M., SATZKE, C., MULHOLLAND, E. K. & LICCIARDI, P. V. 2015. Reduced IL-17A Secretion Is Associated with High Levels of Pneumococcal Nasopharyngeal Carriage in Fijian Children. *Plos One,* 10.

HOLLINGSHEAD, S. K., BECKER, R. & BRILES, D. E. 2000. Diversity of PspA: Mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infection and Immunity,* 68**,** 5889-5900.

HOLMES, A. R., MCNAB, R., MILLSAP, K. W., ROHDE, M., HAMMERSCHMIDT, S., MAWDSLEY, J. L. & JENKINSON, H. F. 2001. The pavA gene of *Streptococcus pneumoniae* encodes a fibronectin-binding protein that is essential for virulence. *Molecular Microbiology,* 41**,** 1395-1408.

HOSTETTER, M. K. 1986. Serotypic variations among virulent pneumococci in deposition and degradation of covalently bound C3B - implications for phagocytosis and antibody-production. *Journal of Infectious Diseases,* 153**,** 682-693.

HUBER, S., SCHRAMM, C., LEHR, H. A., MANN, A., SCHMITT, S., BECKER, C., PROTSCHKA, M., GALLE, P. R., NEURATH, M. F. & BLESSING, M. 2004. Cutting edge: TGF-beta signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory CD4+CD25+ T cells. *J Immunol,* 173**,** 6526-31.

HUSS, A., SCOTT, P., STUCK, A. E., TROTTER, C. & EGGER, M. 2009. Efficacy of pneumococcal vaccination in adults: a meta-analysis. *Canadian Medical Association Journal,* 180**,** 48-58.

HYAMS, C., CAMBERLEIN, E., COHEN, J. M., BAX, K. & BROWN, J. S. 2010a. The *Streptococcus pneumoniae* Capsule Inhibits Complement Activity and Neutrophil Phagocytosis by Multiple Mechanisms. *Infection and Immunity,* 78**,** 704-715.

HYAMS, C., YUSTE, J., BAX, K., CAMBERLEIN, E., WEISER, J. N. & BROWN, J. S. 2010b. *Streptococcus pneumoniae* Resistance to Complement-Mediated Immunity Is Dependent on the Capsular Serotype. *Infection and Immunity,* 78**,** 716-725.

IP, P. P., NIJMAN, H. W. & DAEMEN, T. 2015. Epitope Prediction Assays Combined with Validation Assays Strongly Narrows down Putative Cytotoxic T Lymphocyte Epitopes. *Vaccines,* 3**,** 203-220.

JAIN, S., SELF, W. H., WUNDERINK, R. G., FAKHRAN, S., BALK, R., BRAMLEY, A. M., REED, C., GRIJALVA, C. G., ANDERSON, E. J., COURTNEY, D. M., CHAPPELL, J. D., QI, C., HART, E. M., CARROLL, F., TRABUE, C., DONNELLY, H. K., WILLIAMS, D. J., ZHU, Y., ARNOLD, S. R., AMPOFO, K., WATERER, G. W., LEVINE, M., LINDSTROM, S., WINCHELL, J. M., KATZ, J. M., ERDMAN, D., SCHNEIDER, E., HICKS, L. A., MCCULLERS, J. A., PAVIA, A. T., EDWARDS, K. M., FINELLI, L. & TEAM, C. E. S. 2015a. Community-Acquired Pneumonia Requiring Hospitalization among US Adults. *New England Journal of Medicine,* 373**,** 415-427.

JAIN, S., WILLIAMS, D. J., ARNOLD, S. R., AMPOFO, K., BRAMLEY, A. M., REED, C., STOCKMANN, C., ANDERSON, E. J., GRIJALVA, C. G., SELF, W. H., ZHU, Y. W., PATEL, A., HYMAS, W., CHAPPELL, J. D., KAUFMAN, R. A., KAN, J. H., DANSIE, D., LENNY, N., HILLYARD, D. R., HAYNES, L. M., LEVINE, M., LINDSTROM, S., WINCHELL, J. M., KATZ, J. M., ERDMAN, D., SCHNEIDER, E., HICKS, L. A., WUNDERINK, R. G., EDWARDS, K. M., PAVIA, A. T., MCCULLERS, J. A., FINELLI,

L. & TEAM, C. E. S. 2015b. Community-Acquired Pneumonia Requiring Hospitalization among US Children. *New England Journal of Medicine,* 372**,** 835-845.
JAMBO, K. C., SEPAKO, E., FULLERTON, D. G., MZINZA, D., GLENNIE, S., WRIGHT, A. K., HEYDERMAN, R. S. & GORDON, S. B. 2011. Bronchoalveolar CD4(+) T cell responses to respiratory antigens are impaired in HIV-infected adults. *Thorax,* 66**,** 375-382.
JANOFF, E. N., RUBINS, J. B., FASCHING, C., CHARBONEAU, D., RAHKOLA, J. T., PLAUT, A. G. & WEISER, J. N. 2014. Pneumococcal IgA1 protease subverts specific protection by human IgA1. *Mucosal Immunology,* 7**,** 249-256.
JARVA, H., JANULCZYK, R., HELLWAGE, J., ZIPFEL, P. F., BJORCK, L. & MERI, S. 2002. *Streptococcus pneumoniae* evades complement attack and opsonophagocytosis by expressing the pspC locus-encoded Hic protein that binds to short consensus repeats 8-11 of factor H. *Journal of Immunology,* 168**,** 1886-1894.
JEFFERIES, J. M., MACDONALD, E., FAUST, S. N. & CLARKE, S. C. 2011. 13-valent pneumococcal conjugate vaccine (PCV13). *Hum Vaccin,* 7**,** 1012-8.
JIANG, X. L., ZHANG, G. L., YANG, T., YANG, B. H., WANG, L. J., WANG, Q. H., LUO, Z. X., LIU, E. M. & FU, Z. 2015. Association of Pneumococcal Carriage and Expression of Foxp3+ Regulatory T Cells and Th17 Cells in the Adenoids of Children. *Respiration,* 90**,** 25-32.
JOCHEMS, S. P., PIDDOCK, K., RYLANCE, J., ADLER, H., CARNIEL, B. F., COLLINS, A., GRITZFELD, J. F., HANCOCK, C., HILL, H., REINE, J., SEDDON, A., SOLORZANO, C., SUNNY, S., TRIMBLE, A., WRIGHT, A. D., ZAIDI, S., GORDON, S. B. & FERREIRA, D. M. 2017. Novel Analysis of Immune Cells from Nasal Microbiopsy Demonstrates Reliable, Reproducible Data for Immune Populations, and Superior Cytokine Detection Compared to Nasal Wash. *PLoS One,* 12**,** e0169805.
JONSSON, S., MUSHER, D. M., CHAPMAN, A., GOREE, A. & LAWRENCE, E. C. 1985. Phagocytosis and killing of common bacterial pathogens of the lung by human alveolar macrophages. *Journal of Infectious Diseases,* 152**,** 4-13.
KANG, C. I., ROUSE, M. S., PATEL, R., KITA, H. & JUHN, Y. J. 2009. Allergic airway inflammation and susceptibility to pneumococcal pneumonia in a murine model with real-time in vivo evaluation. *Clin Exp Immunol,* 156**,** 552-61.
KATSURA, H., PIAO, Z., IWATSUKI-HORIMOTO, K., AKEDA, Y., WATANABE, S., HORIMOTO, T., OISHI, K. & KAWAOKA, Y. 2014. A Bivalent Vaccine Based on a Replication-Incompetent Influenza Virus Protects against *Streptococcus pneumoniae* and Influenza Virus Infection. *Journal of Virology,* 88**,** 13410-13417.
KEMP, K., BRUUNSGAARD, H., SKINHOJ, P. & KLARLUND PEDERSEN, B. 2002. Pneumococcal infections in humans are associated with increased apoptosis and trafficking of type 1 cytokine-producing T cells. *Infect Immun,* 70**,** 5019-25.
KERR, A. R., PATERSON, G. K., MCCLUSKEY, J., IANNELLI, F., OGGIONI, M. R., POZZI, G. & MITCHELL, T. J. 2006. The contribution of PspC to pneumococcal virulence varies between strains and is accomplished by both complement evasion and complement-independent mechanisms. *Infection and Immunity,* 74**,** 5319-5324.
KHAN, M. N., SHARMA, S. K., FILKINS, L. M. & PICHICHERO, M. E. 2012. PcpA of *Streptococcus pneumoniae* mediates adherence to nasopharyngeal and lung epithelial cells and elicits functional antibodies in humans. *Microbes and Infection,* 14**,** 1102-1110.
KIM, J. O. & WEISER, J. N. 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. *J Infect Dis,* 177**,** 368-77.
KIRBY, A. C., RAYNES, J. G. & KAYE, P. M. 2005. The role played by tumor necrosis factor during localized and systemic infection with *Streptococcus pneumoniae*. *J Infect Dis,* 191**,** 1538-47.
KLEIN, J. O., PEDIATRIC INFECTIOUS DISEASES, B. M. C., MASSACHUSETTS, PLOTKIN, S. A., UNIVERSITY OF PENNSYLVANIA, D., PENNSYLVANIA & SANOFI PASTEUR, D., PENNSYLVANIA 2017. Robert Austrian: 1917–2007. *Clinical Infectious Diseases,* 45**,** 2-3.
KOBE, B. & KAJAVA, A. V. 2001. The leucine-rich repeat as a protein recognition motif. *Current Opinion in Structural Biology,* 11**,** 725-732.

KOLBERG, J., AASE, A., MICHAELSEN, T. E. & RODAL, G. 2001. Epitope analyses of pneumococcal surface protein A: a combination of two monoclonal antibodies detects 94% of clinical isolates. *Fems Immunology and Medical Microbiology,* 31**,** 175-180.

KOLBERG, J., AASE, A., RODAL, G., LITTLEJOHN, J. E. & JEDRZEJAS, M. J. 2003. Epitope mapping of pneumococcal surface protein A of strain Rx1 using monoclonal antibodies and molecular structure modelling. *Fems Immunology and Medical Microbiology,* 39**,** 265-273.

KOTHARI, N., GENSCHMER, K. R., KOTHARI, S., KIM, J. A., BRILES, D. E., RHEE, D. K. & CARBIS, R. 2014. Preparation and testing of a Vi conjugate vaccine using pneumococcal surface protein A (PspA) from *Streptococcus pneumoniae* as the carrier protein. *Vaccine,* 32**,** 5755-5760.

KOTHARI, N., KOTHARI, S., CHOI, Y. J., DEY, A., BRILES, D. E., RHEE, D. K. & CARBIS, R. 2015. A bivalent conjugate vaccine containing PspA families 1 and 2 has the potential to protect against a wide range of *Streptococcus pneumoniae* strains and Salmonella Typhi. *Vaccine,* 33**,** 783-788.

KRISHNAN, J., SELVARAJOO, K., TSUCHIYA, M., LEE, G. & CHOI, S. 2007. Toll-like receptor signal transduction. *Exp Mol Med,* 39**,** 421-38.

KUIPERS, K., DALEKE-SCHERMERHORN, M. H., JONG, W. S., TEN HAGEN-JONGMAN, C. M., VAN OPZEELAND, F., SIMONETTI, E., LUIRINK, J. & DE JONGE, M. I. 2015. Salmonella outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonization. *Vaccine,* 33**,** 2022-9.

KUMAR, H., KAWAI, T. & AKIRA, S. 2011. Pathogen Recognition by the Innate Immune System. *International Reviews of Immunology,* 30**,** 16-34.

KUMAR, S., VERMA, A. K., SHARMA, A., KUMAR, D., TRIPATHI, A., CHAUDHARI, B. P., DAS, M., JAIN, S. K. & DWIVEDI, P. D. 2013. Phytohemagglutinins augment red kidney bean (Phaseolus vulgaris L.) induced allergic manifestations. *Journal of Proteomics,* 93**,** 50-64.

LAGOUSI, T., ROUTSIAS, J., PIPERI, C., TSAKRIS, A., CHROUSOS, G., THEODORIDOU, M. & SPOULOU, V. 2015. Discovery of Immunodominant B Cell Epitopes within Surface Pneumococcal Virulence Proteins in Pediatric Patients with Invasive Pneumococcal Disease. *Journal of Biological Chemistry,* 290**,** 27500-27510.

LAZAR, I. 2017. [Online]. Available at: <http://www.GelAnalyzer.com> [Accessed February 2017].

LEDERMAN, H. M. & WINKELSTEIN, J. A. 1985. X-linked agammaglobulinemia: an analysis of 96 patients. *Medicine (Baltimore),* 64**,** 145-56.

LEE, K. S., SCANGA, C. A., BACHELDER, E. M., CHEN, Q. Y. & SNAPPER, C. M. 2007. TLR2 synergizes with both TLR4 and TLR9 for induction of the MyD88-dependent splenic cytokine and chemokine response to *Streptococcus pneumoniae*. *Cellular Immunology,* 245**,** 103-110.

LEFEBER, D. J., BENAISSA-TROUW, B., VLIEGENTHART, J. F., KAMERLING, J. P., JANSEN, W. T., KRAAIJEVELD, K. & SNIPPE, H. 2003. Th1-directing adjuvants increase the immunogenicity of oligosaccharide-protein conjugate vaccines related to *Streptococcus pneumoniae* type 3. *Infect Immun,* 71**,** 6915-20.

LEMESSURIER, K. S., OGUNNIYI, A. D. & PATON, J. C. 2006. Differential expression of key pneumococcal virulence genes in vivo. *Microbiology-Sgm,* 152**,** 305-311.

LI, Q., LI, Y. X., STAHL, G. L., THURMAN, J. M., HE, Y. J. & TONG, H. H. 2011. Essential Role of Factor B of the Alternative Complement Pathway in Complement Activation and Opsonophagocytosis during Acute Pneumococcal Otitis Media in Mice. *Infection and Immunity,* 79**,** 2578-2585.

LI, S. L., KELLY, S. J., LAMANI, E., FERRARONI, M. & JEDRZEJAS, M. J. 2000. Structural basis of hyaluronan degradation by *Streptococcus pneumoniae* hyaluronate lyase. *Embo Journal,* 19**,** 1228-1240.

LU, J., SUN, T., WANG, D., DONG, Y., XU, M., HOU, H., KONG, F. T., LIANG, C., GU, T., CHEN, P., SUN, S., LV, X., JIANG, C., KONG, W. & WU, Y. 2015. Protective Immune Responses Elicited by Fusion Protein Containing PsaA and PspA Fragments. *Immunological Investigations,* 44**,** 482-496.

LU, Y. J., GROSS, J., BOGAERT, D., FINN, A., BAGRADE, L., ZHANG, Q., KOLLS, J. K., SRIVASTAVA, A., LUNDGREN, A., FORTE, S., THOMPSON, C. M., HARNEY, K. F., ANDERSON, P. W., LIPSITCH, M. & MALLEY, R. 2008. Interleukin-17A mediates acquired immunity to pneumococcal colonization. *Plos Pathogens,* 4.

LUNDGREN, A., BHUIYAN, T. R., NOVAK, D., KAIM, J., RESKE, A., LU, Y. J., QADRI, F. & MALLEY, R. 2012. Characterization of Th17 responses to *Streptococcus pneumoniae* in humans: comparisons between adults and children in a developed and a developing country. *Vaccine,* 30**,** 3897-907.

MACLEOD, C. M., HODGES, R. G., HEIDELBERGER, M. & BERNHARD, W. G. 1945. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *J Exp Med.* 30, 445-65.

MACMILLAN, H., NORIMINE, J., BRAYTON, K. A., PALMER, G. H. & BROWN, W. C. 2008. Physical linkage of naturally complexed bacterial outer membrane proteins enhances immunogenicity. *Infect Immun,* 76**,** 1223-9.

MAHDI, L. K., WANG, H., VAN DER HOEK, M. B., PATON, J. C. & OGUNNIYI, A. D. 2012. Identification of a novel pneumococcal vaccine antigen preferentially expressed during meningitis in mice. *J Clin Invest,* 122**,** 2208-20.

MALHERBE, L. 2009. T-cell epitope mapping. *Ann Allergy Asthma Immunol,* 103**,** 76-9.

MALLEY, R., HENNEKE, P., MORSE, S. C., CIESLEWICZ, M. J., LIPSITCH, M., THOMPSON, C. M., KURT-JONES, E., PATON, J. C., WESSELS, M. R. & GOLENBOCK, D. T. 2003. Recognition of pneumolysin by toll-like receptor 4 confers resistance to pneumococcal infection. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 1966-1971.

MALLEY, R., LIPSITCH, M., STACK, A., SALADINO, R., FLEISHER, G., PELTON, S., THOMPSON, C., BRILES, D. & ANDERSON, P. 2001. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. *Infection and Immunity,* 69**,** 4870-4873.

MALLEY, R., MORSE, S. C., LEITE, L. C. C., AREAS, A. P. M., HO, P. L., KUBRUSLY, F. S., ALMEIDA, I. C. & ANDERSON, P. 2004. Multiserotype protection of mice against pneumococcal colonization of the nasopharynx and middle ear by killed nonencapsulated cells given intranasally with a nontoxic adjuvant. *Infection and Immunity,* 72**,** 4290-4292.

MALLEY, R., SRIVASTAVA, A., LIPSITCH, M., THOMPSON, C. M., WATKINS, C., TZIANABOS, A. & ANDERSON, P. W. 2006. Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. *Infection and Immunity,* 74**,** 2187-2195.

MALLEY, R., TRZCIŃSKI , K., SRIVASTAVA, A., THOMPSON, C. M., ANDERSON, P. W. & LIPSITCH, M. 2005. CD4(+) T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 4848-4853.

MARQUES, J. M., RIAL, A., MUNOZ, N., PELLAY, F. X., MAELE, L., LEGER, H., CAMOU, T., SIRARD, J. C., BENECKE, A. & CHABALGOITY, J. A. 2012. Protection against *Streptococcus pneumoniae* serotype 1 acute infection shows a signature of Th17-and IFN-gamma-mediated immunity. *Immunobiology,* 217**,** 420-429.

MCCOOL, T. L., CATE, T. R., MOY, G. & WEISER, J. N. 2002. The immune response to pneumococcal proteins during experimental human carriage. *Journal of Experimental Medicine,* 195**,** 359-365.

MCCOOL, T. L. & WEISER, J. N. 2004. Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. *Infection and Immunity,* 72**,** 5807-5813.

MCDANIEL, L. S., RALPH, B. A., MCDANIEL, D. O. & BRILES, D. E. 1994. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residue 192 and residue 260. *Microbial Pathogenesis,* 17**,** 323-337.

MCDANIEL, L. S., SCOTT, G., KEARNEY, J. F. & BRILES, D. E. 1984. Monoclonal antibodies against protease sensitive pneumococcal antigens can protect mice from fatal infection with *Streptococcus pneumoniae*. *Journal of Experimental Medicine,* 160**,** 386-397.

MCDANIEL, L. S., SCOTT, G., WIDENHOFER, K., CARROLL, J. M. & BRILES, D. E. 1986. Analysis of a surface protein of *Streptococcus pneumoniae* recognized by protective monoclonal antibodies. *Microbial Pathogenesis,* 1**,** 519-531.

MCENTYRE, J. 1998. Linking up with Entrez. *Trends Genet,* 14**,** 39-40.

MCGUIRK, P., MCCANN, C. & MILLS, K. H. 2002. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10

production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis. *J Exp Med,* 195**,** 221-31.

MCNEELA, E. A., BURKE, A., NEILL, D. R., BAXTER, C., FERNANDES, V. E., FERREIRA, D., SMEATON, S., EL-RACHKIDY, R., MCLOUGHLIN, R. M., MORI, A., MORAN, B., FITZGERALD, K. A., TSCHOPP, J., PETRILLI, V., ANDREW, P. W., KADIOGLU, A. & LAVELLE, E. C. 2010. Pneumolysin Activates the NLRP3 Inflammasome and Promotes Proinflammatory Cytokines Independently of TLR4. *Plos Pathogens,* 6.

MELIN, M., COAN, P. & HOLLINGSHEAD, S. 2012. Development of cross-reactive antibodies to the proline-rich region of pneumococcal surface protein A in children. *Vaccine,* 30**,** 7157-7160.

MELIN, M., TRZCIŃSKI , K., ANTONIO, M., MERI, S., ADEGBOLA, R., KAIJALAINEN, T., KAYHTY, H. & VAKEVAINEN, M. 2010. Serotype-Related Variation in Susceptibility to Complement Deposition and Opsonophagocytosis among Clinical Isolates of *Streptococcus pneumoniae*. *Infection and Immunity,* 78**,** 5252-5261.

MILES, A. A., MISRA, S. S. & IRWIN, J. O. 1938. The estimation of the bactericidal power of the blood. *Journal of Hygiene,* 38**,** 732-749.

MIRZA, S., WILSON, L., BENJAMIN, W. H., NOVAK, J., BARNES, S., HOLLINGSHEAD, S. K. & BRILES, D. E. 2011. Serine Protease PrtA from *Streptococcus pneumoniae* Plays a Role in the Killing of S. pneumoniae by Apolactoferrin. *Infection and Immunity,* 79**,** 2440-2450.

MITCHELL, T. J. & DALZIEL, C. E. 2014. The biology of pneumolysin. *Subcell Biochem,* 80**,** 145-60.

MITSI, E., ROCHE, A. M., REINE, J., ZANGARI, T., OWUGHA, J. T., PENNINGTON, S. H., GRITZFELD, J. F., WRIGHT, A. D., COLLINS, A. M., VAN SELM, S., DE JONGE, M. I., GORDON, S. B., WEISER, J. N. & FERREIRA, D. M. 2017. Agglutination by anti-capsular polysaccharide antibody is associated with protection against experimental human pneumococcal carriage. *Mucosal Immunol,* 10**,** 385-394.

MIYAJI, E. N., FERREIRA, D. M., LOPES, A. P. Y., BRANDILEONE, M. C. C., DIAS, W. O. & LEITE, L. C. C. 2002. Analysis of serum cross-reactivity and cross-protection elicited by immunization with DNA vaccines against *Streptococcus pneumoniae* expressing PspA fragments from different clades. *Infection and Immunity,* 70**,** 5086-5090.

MIYAJI, E. N., OLIVEIRA, M. L. S., CARVALHO, E. & HO, P. L. 2013. Serotype-independent pneumococcal vaccines. *Cellular and Molecular Life Sciences,* 70**,** 3303-3326.

MIYAJI, E. N., VADESILHO, C. F. M., OLIVEIRA, M. L. S., ZELANIS, A., BRILES, D. E. & HO, P. L. 2015. Evaluation of a Vaccine Formulation against *Streptococcus pneumoniae* Based on Choline-Binding Proteins. *Clinical and Vaccine Immunology,* 22**,** 213-220.

MIZGERD, J. P. 2012. Respiratory Infection and the Impact of Pulmonary Immunity on Lung Health and Disease. *American Journal of Respiratory and Critical Care Medicine,* 186**,** 824-829.

MOFFITT, K., SKOBERNE, M., HOWARD, A., GAVRILESCU, C., GIERAHN, T., MUNZER, S., DIXIT, B., GIANNASCA, P., FLECHTNER, J. B. & MALLEY, R. 2014. Toll-Like Receptor 2-Dependent Protection against Pneumococcal Carriage by Immunization with Lipidated Pneumococcal Proteins. *Infection and Immunity,* 82**,** 2079-2086.

MOFFITT, K. L., GIERAHN, T. M., LU, Y.-J., GOUVEIA, P., ALDERSON, M., FLECHTNER, J. B., HIGGINS, D. E. & MALLEY, R. 2011. T(H)17-Based Vaccine Design for Prevention of *Streptococcus pneumoniae* Colonization. *Cell Host & Microbe,* 9**,** 158-165.

MOGENSEN, T. H., PALUDAN, S. R., KILIAN, M. & OSTERGAARD, L. 2006. Live *Streptococcus pneumoniae*, Haemophilus influenzae, and Neisseria meningitidis activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. *Journal of Leukocyte Biology,* 80**,** 267-277.

MORENO, A. T., OLIVEIRA, M. L. S., FERREIRA, D. M., HO, P. L., DARRIEUX, M., LEITE, L. C. C., FERREIRA, J. M. C., JR., PIMENTA, F. C., ANDRADE, A. L. S. S. & MIYAJI, E. N. 2010. Immunization of Mice with Single PspA Fragments Induces Antibodies Capable of Mediating Complement Deposition on Different Pneumococcal Strains and Cross-Protection. *Clinical and Vaccine Immunology,* 17**,** 439-446.

MORENO, A. T., OLIVEIRA, M. L. S., HO, P. L., VADESILHO, C. F. M., PALMA, G. M. P., FERREIRA, J. M. C., JR., FERREIRA, D. M., SANTOS, S. R., MARTINEZ, M. B. & MIYAJI, E. N. 2012. Cross-Reactivity of Antipneumococcal Surface Protein C (PspC) Antibodies with Different Strains and Evaluation of Inhibition of Human Complement

Factor H and Secretory IgA Binding via PspC. *Clinical and Vaccine Immunology,* 19**,** 499-507.

MORTIMER, K., NDAMALA, C. B., NAUNJE, A. W., MALAVA, J., KATUNDU, C., WESTON, W., HAVENS, D., POPE, D., BRUCE, N. G., NYIRENDA, M., WANG, D. L., CRAMPIN, A., GRIGG, J., BALMES, J. & GORDON, S. B. 2017. A cleaner burning biomass-fuelled cookstove intervention to prevent pneumonia in children under 5 years old in rural Malawi (the Cooking and Pneumonia Study): a cluster randomised controlled trial. *Lancet,* 389**,** 167-175.

MORTON, B., MITSI, E., PENNINGTON, S. H., REINE, J., WRIGHT, A. D., PARKER, R., WELTERS, I. D., BLAKEY, J. D., RAJAM, G., ADES, E. W., FERREIRA, D. M., WANG, D., KADIOGLU, A. & GORDON, S. B. 2016. Augmented passive immunotherapy with P4 peptide improves phagocyte activity in severe sepsis. *Shock,* 46**,** 635-641.

MORTON, B., PENNINGTON, S. H. & GORDON, S. B. 2014. Immunomodulatory adjuvant therapy in severe community-acquired pneumonia. *Expert Rev. Respir. Med.* 1–10.

MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol,* 8**,** 958-69.

MUBARAK, A., AHMED, M. S., UPILE, N., VAUGHAN, C., XIE, C., SHARMA, R., ACAR, P., MCCORMICK, M. S., PATON, J. C., MITCHELL, T., CUNLIFFE, N. & ZHANG, Q. 2016. A dynamic relationship between mucosal T helper type 17 and regulatory T-cell populations in nasopharynx evolves with age and associates with the clearance of pneumococcal carriage in humans. *Clin Microbiol Infect,* 22**,** 736.e1-7.

MUKERJI, R., MIRZA, S., ROCHE, A. M., WIDENER, R. W., CRONEY, C. M., RHEE, D. K., WEISER, J. N., SZALAI, A. J. & BRILES, D. E. 2012. Pneumococcal Surface Protein A Inhibits Complement Deposition on the Pneumococcal Surface by Competing with the Binding of C-Reactive Protein to Cell-Surface Phosphocholine. *Journal of Immunology,* 189**,** 5327-5335.

MURPHY, K. & WEAVER, C. 2017. Janeway's Immunobiology, 9th Edition. *Janeway's Immunobiology, 9th Edition***,** 1-904.

MUSHER, D. M. & THORNER, A. R. 2014. Community-acquired pneumonia. *N Engl J Med,* 371**,** 1619-28.

NABORS, G. S., BRAUN, P. A., HERRMANN, D. J., HEISE, M. L., PYLE, D. J., GRAVENSTEIN, S., SCHILLING, M., FERGUSON, L. M., HOLLINGSHEAD, S. K., BRILES, D. E. & BECKER, R. S. 2000. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. *Vaccine,* 18**,** 1743-1754.

NEILL, D. R., COWARD, W. R., GRITZFELD, J. F., RICHARDS, L., GARCIA-GARCIA, F. J., DOTOR, J., GORDON, S. B. & KADIOGLU, A. 2014. Density and Duration of Pneumococcal Carriage Is Maintained by Transforming Growth Factor beta 1 and T Regulatory Cells. *American Journal of Respiratory and Critical Care Medicine,* 189**,** 1250-1259.

NEILL, D. R., FERNANDES, V. E., WISBY, L., HAYNES, A. R., FERREIRA, D. M., LAHER, A., STRICKLAND, N., GORDON, S. B., DENNY, P., KADIOGLU, A. & ANDREW, P. W. 2012. T Regulatory Cells Control Susceptibility to Invasive Pneumococcal Pneumonia in Mice. *Plos Pathogens,* 8.

NELSON, A. L., RIES, J., BAGNOLI, F., DAHLBERG, S., FALKER, S., ROUNIOJA, S., TSCHOP, J., MORFELDT, E., FERLENGHI, I., HILLERINGMANN, M., HOLDEN, D. W., RAPPUOLI, R., NORMARK, S., BAROCCHI, M. A. & HENRIQUES-NORMARK, B. 2007. RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae*. *Molecular Microbiology,* 66**,** 329-340.

NISINI, R., PAROLI, M., ACCAPEZZATO, D., BONINO, F., ROSINA, F., SANTANTONIO, T., SALLUSTO, F., AMOROSO, A., HOUGHTON, M. & BARNABA, V. 1997. Human CD4+ T-cell response to hepatitis delta virus: identification of multiple epitopes and characterization of T-helper cytokine profiles. *J Virol,* 71**,** 2241-51.

NIU, S. Q., LUO, M., TANG, J., ZHOU, H., ZHANG, Y. L., MIN, X., CAI, X. F., ZHANG, W. L., XU, W. C., LI, D. F., DING, J. J., HU, Y. L., WANG, D. C., HUANG, A. L., YIN, Y. B. & WANG, D. Q. 2013. Structural Basis of the Novel S. pneumoniae Virulence Factor, GHIP, a Glycosyl Hydrolase 25 Participating in Host-Cell Invasion. *Plos One,* 8.

OCHS, H. D. & THRASHER, A. J. 2006. The Wiskott-Aldrich syndrome. *J Allergy Clin Immunol,* 117**,** 725-38; quiz 739.

OCHS, M. M., BARTLETT, W., BRILES, D. E., HICKS, B., JURKUVENAS, A., LAU, P., REN, B. & MILLAR, A. 2008. Vaccine-induced human antibodies to PspA augment complement C3 deposition on *Streptococcus pneumoniae*. *Microbial Pathogenesis,* 44**,** 204-214.

OGGIONI, M. R., MEMMI, G., MAGGI, T., CHIAVOLINI, D., IANNELLI, F. & POZZI, G. 2003. Pneumococcal zinc metalloproteinase ZmpC cleaves human matrix metalloproteinase 9 and is a virulence factor in experimental pneumonia. *Mol Microbiol,* 49**,** 795-805.

OGGIONI, M. R., TRAPPETTI, C., KADIOGLU, A., CASSONE, M., IANNELLI, F., RICCI, S., ANDREW, P. W. & POZZI, G. 2006. Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. *Molecular Microbiology,* 61**,** 1196-1210.

OGUNNIYI, A. D., GRABOWICZ, M., MAHDI, L. K., COOK, J., GORDON, D. L., SADLON, T. A. & PATON, J. C. 2009. Pneumococcal histidine triad proteins are regulated by the Zn2+-dependent repressor AdcR and inhibit complement deposition through the recruitment of complement factor H. *Faseb Journal,* 23**,** 731-738.

OLAFSDOTTIR, T. A., LINGNAU, K., NAGY, E. & JONSDOTTIR, I. 2012. Novel protein-based pneumococcal vaccines administered with the Th1-promoting adjuvant IC31 induce protective immunity against pneumococcal disease in neonatal mice. *Infect Immun,* 80**,** 461-8.

OLIVEIRA, M. L. S., MONEDERO, V., MIYAJI, E. N., LEITE, L. C. C., HO, P. L. & PEREZ-MARTINEZ, G. 2003. Expression of *Streptococcus pneumoniae* antigens, PsaA (pneumococcal surface antigen A) and PspA (pneumococcal surface protein A) by Lactobacillus casei. *Fems Microbiology Letters,* 227**,** 25-31.

OLLIVER, M., HIEW, J., MELLROTH, P., HENRIQUES-NORMARK, B. & BERGMAN, P. 2011. Human monocytes promote Th1 and Th17 responses to *Streptococcus pneumoniae*. *Infect Immun,* 79**,** 4210-7.

ORIHUELA, C. J., GAO, G. L., FRANCIS, K. P., YU, J. & TUOMANEN, E. I. 2004. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *Journal of Infectious Diseases,* 190**,** 1661-1669.

OUKKA, M. 2008. Th17 cells in immunity and autoimmunity. *Annals of the Rheumatic Diseases,* 67**,** 26-29.

OUYANG, W. J., KOLLS, J. K. & ZHENG, Y. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity,* 28**,** 454-467.

PANIGADA, M., STURNIOLO, T., BESOZZI, G., BOCCIERI, M. G., SINIGAGLIA, F., GRASSI, G. G. & GRASSI, F. 2002. Identification of a promiscuous T-cell epitope in Mycobacterium tuberculosis Mce proteins. *Infect Immun,* 70**,** 79-85.

PAOLETTI, L. C., KASPER, D. L., MICHON, F., DIFABIO, J., HOLME, K., JENNINGS, H. J. & WESSELS, M. R. 1990. An oligosaccharide tetanus toxoid conjugate vaccine against type iii group b streptococcus. *Journal of Biological Chemistry,* 265**,** 18278-18283.

PARISH, C. R. 1999. Fluorescent dyes for lymphocyte migration and proliferation studies. *Immunology and Cell Biology,* 77**,** 499-508.

PATERSON, G. K. & ORIHUELA, C. J. 2010. Pneumococci: immunology of the innate host response. *Respirology,* 15**,** 1057-1063.

PECK, A. & MELLINS, E. D. 2010. Precarious Balance: Th17 Cells in Host Defense. *Infect Immun,* 78**,** 32-8.

PENNINGTON, S. H., POJAR, S., MITSI, E., GRITZFELD, J. F., NIKOLAOU, E., SOLORZANO, C., OWUGHA, J. T., MASOOD, Q., GORDON, M. A., WRIGHT, A. D., COLLINS, A. M., MIYAJI, E. N., GORDON, S. B. & FERREIRA, D. M. 2016. Polysaccharide-Specific Memory B Cells Predict Protection against Experimental Human Pneumococcal Carriage. *American Journal of Respiratory and Critical Care Medicine,* 194**,** 1523-1531.

PICARD, C. & CASANOVA, J. L. 2003. New primary immunodeficiency with infectious disease genetic predisposition. *Archives De Pediatrie,* 10**,** 513S-516S.

PIDO-LOPEZ, J., KWOK, W. W., MITCHELL, T. J., HEYDERMAN, R. S. & WILLIAMS, N. A. 2011. Acquisition of Pneumococci Specific Effector and Regulatory Cd4(+) T Cells Localising within Human Upper Respiratory-Tract Mucosal Lymphoid Tissue. *Plos Pathogens,* 7.

PNEUMOVAX, 2017. *Products* [Online]. Available at: <https://www.merckvaccines.com/Products/Pneumovax> [Accessed December 2016].

PRACHT, D., ELM, C., GERBER, J., BERGMANN, S., ROHDE, M., SEILER, M., KIM, K. S., JENKINSON, H. F., NAU, R. & HAMMERSCHMIDT, S. 2005. PavA of *Streptococcus pneumoniae* modulates adherence, invasion, and meningeal inflammation. *Infection and Immunity,* 73**,** 2680-2689.

PRICKETT, S. R., VOSKAMP, A. L., PHAN, T., DACUMOS-HILL, A., MANNERING, S. I., ROLLAND, J. M. & O'HEHIR, R. E. 2013. Ara h 1 CD4+ T cell epitope-based peptides: candidates for a peanut allergy therapeutic. *Clin Exp Allergy,* 43**,** 684-97.

PURWAR, R., CAMPBELL, J., MURPHY, G., RICHARDS, W. G., CLARK, R. A. & KUPPER, T. S. 2011. Resident Memory T Cells (T-RM) Are Abundant in Human Lung: Diversity, Function, and Antigen Specificity. *Plos One,* 6.

QUAH, B. J., WARREN, H. S. & PARISH, C. R. 2007. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc,* 2**,** 2049-56.

RAJAM, G., PHILLIPS, D. J., WHITE, E., ANDERTON, J., HOOPER, C. W., SAMPSON, J. S., CARLONE, G. M., ADES, E. W. & ROMERO-STEINER, S. 2008. A functional epitope of the pneumococcal surface adhesin A activates nasopharyngeal cells and increases bacterial internalization. *Microbial Pathogenesis,* 44**,** 186-196.

RAMOS, C. R. R., ABREU, P. A. E., NASCIMENTO, A. & HO, P. L. 2004. A high-copy T7 Escherichia coli expression vector for the production of recombinant proteins with a minimal N-terminal his-tagged fusion peptide. *Brazilian Journal of Medical and Biological Research,* 37**,** 1103-1109.

RAMOS-SEVILLANO, E., MOSCOSO, M., GARCIA, P., GARCIA, E. & YUSTE, J. 2011. Nasopharyngeal Colonization and Invasive Disease Are Enhanced by the Cell Wall Hydrolases LytB and LytC of *Streptococcus pneumoniae*. *Plos One,* 6.

RAPOLA, S., JANTTI, V., HAIKALA, R., SYRJANEN, R., CARLONE, G. M., SAMPSON, J. S., BRILES, D. E., PATON, J. C., TAKALA, A. K., KILPI, T. M. & KAYHTY, H. 2000. Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. *Journal of Infectious Diseases,* 182**,** 1146-1152.

RATNER, A. J., HIPPE, K. R., AGUILAR, J. L., BENDER, M. H., NELSON, A. L. & WEISER, J. N. 2006. Epithelial cells are sensitive detectors of bacterial pore-forming toxins. *Journal of Biological Chemistry,* 281**,** 12994-12998.

RAVKOV, E. V. & WILLIAMS, M. A. 2009. The magnitude of CD4+ T cell recall responses is controlled by the duration of the secondary stimulus. *J Immunol,* 183**,** 2382-9.

REGEV-YOCHAY, G., RAZ, M., DAGAN, R., PORAT, N., SHAINBERG, B., PINCO, E., KELLER, N. & RUBINSTEIN, E. 2004. Nasopharyngeal carriage of *Streptococcus pneumoniae* by adults and children in community and family settings. *Clinical Infectious Diseases,* 38**,** 632-639.

REISMAN, J., RUDOLPH, K., BRUDEN, D., HURLBURT, D., BRUCE, M. G. & HENNESSY, T. 2014. Risk Factors for Pneumococcal Colonization of the Nasopharynx in Alaska Native Adults and Children. *J Pediatric Infect Dis Soc,* 3**,** 104-11.

REN, B., MCCRORY, M. A., PASS, C., BULLARD, D. C., BALLANTYNE, C. M., XU, Y. Y., BRILES, D. E. & SZALAI, A. J. 2004. The virulence function of *Streptococcus pneumoniae* surface protein A involves inhibition of complement activation and impairment of complement receptor-mediated protection. *Journal of Immunology,* 173**,** 7506-7512.

RICERCA, B. M., DI GIROLAMO, A. & RUND, D. 2009. Infections in thalassemia and hemoglobinopathies: focus on therapy-related complications. *Mediterr J Hematol Infect Dis,* 1**,** e2009028.

RILEY, I. D., TARR, P. I., ANDREWS, M., PFEIFFER, M., HOWARD, R., CHALLANDS, P. & JENNISON, G. 1977. Immunisation with a polyvalent pneumococcal vaccine. Reduction of adult respiratory mortality in a New Guinea Highlands community. *Lancet,* 1**,** 1338-41.

RING, A., WEISER, J. N. & TUOMANEN, E. I. 1998. Pneumococcal trafficking across the blood-brain barrier - Molecular analysis of a novel bidirectional pathway. *Journal of Clinical Investigation,* 102**,** 347-360.

ROCHE, A. M., RICHARD, A. L., RAHKOLA, J. T., JANOFF, E. N. & WEISER, J. N. 2015. Antibody blocks acquisition of bacterial colonization through agglutination. *Mucosal Immunology,* 8**,** 176-185.

ROCHE, H., HAKANSSON, A., HOLLINGSHEAD, S. K. & BRILES, D. E. 2003. Regions of PspA/EF3296 best able to elicit protection against *Streptococcus pneumoniae* in a murine infection model. *Infection and Immunity,* 71**,** 1033-1041.

RODGERS, G. L. & KLUGMAN, K. P. 2016. Surveillance of the impact of pneumococcal conjugate vaccines in developing countries. *Human Vaccines & Immunotherapeutics,* 12**,** 417-420.

RODRIGUES, F., FOSTER, D., NICOLI, E., TROTTER, C., VIPOND, B., MUIR, P., GONCALVES, G., JANUARIO, L. & FINN, A. 2013a. Relationships Between Rhinitis Symptoms, Respiratory Viral Infections and Nasopharyngeal Colonization With *Streptococcus pneumoniae*, Haemophilus influenzae and Staphylococcus aureus in Children Attending Daycare. *Pediatric Infectious Disease Journal,* 32**,** 227-232.

RODRIGUES, F., MORALES-AZA, B., TURNER, K. M. E., SIKORA, P., GOULD, K., HINDS, J., GONCALVES, G., JANUARIO, L. & FINN, A. 2013b. Multiple *Streptococcus pneumoniae* Serotypes in Aural Discharge Samples from Children with Acute Otitis Media with Spontaneous Otorrhea. *Journal of Clinical Microbiology,* 51**,** 3409-3411.

ROEDERER, M. 2011. Interpretation of Cellular Proliferation Data: Avoid the Panglossian. *Cytometry Part A,* 79A**,** 95-101.

ROLSTON, K. V. 2001. The spectrum of pulmonary infections in cancer patients. *Curr Opin Oncol,* 13**,** 218-23.

ROSENOW, C., RYAN, P., WEISER, J. N., JOHNSON, S., FONTAN, P., ORTQVIST, A. & MASURE, H. R. 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Molecular Microbiology,* 25**,** 819-829.

ROZENBAUM, M. H., VAN HOEK, A. J., FLEMING, D., TROTTER, C. L., MILLER, E. & EDMUNDS, W. J. 2012. Vaccination of risk groups in England using the 13 valent pneumococcal conjugate vaccine: economic analysis. *Bmj-British Medical Journal,* 345.

RUBINS, J. B. & POMEROY, C. 1997. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. *Infection and Immunity,* 65**,** 2975-2977.

RUBIO-INFANTE, N., GOVEA-ALONSO, D. O., ROMERO-MALDONADO, A., GARCIA-HERNANDEZ, A. L., ILHUICATZI-ALVARADO, D., SALAZAR-GONZALEZ, J. A., KORBAN, S. S., ROSALES-MENDOZA, S. & MORENO-FIERROS, L. 2015. A Plant-Derived Multi-HIV Antigen Induces Broad Immune Responses in Orally Immunized Mice. *Mol Biotechnol,* 57**,** 662-74.

RUDAN, I., TOMASKOVIC, L., BOSCHI-PINTO, C., CAMPBELL, H. & GRP, W. H. O. C. H. E. R. 2004. Global estimate of the incidence of clinical pneumonia among children under five years of age. *Bulletin of the World Health Organization,* 82**,** 895-903.

SANCHEZ, C. J., SHIVSHANKAR, P., STOL, K., TRAKHTENBROIT, S., SULLAM, P. M., SAUER, K., HERMANS, P. W. M. & ORIHUELA, C. J. 2010. The Pneumococcal Serine-Rich Repeat Protein Is an Intra-Species Bacterial Adhesin That Promotes Bacterial Aggregation In Vivo and in Biofilms. *Plos Pathogens,* 6.

SANCHEZ-BEATO, A. R., LOPEZ, R. & GARCIA, J. L. 1998. Molecular characterization of PcpA: a novel choline-binding protein of *Streptococcus pneumoniae*. *Fems Microbiology Letters,* 164**,** 207-214.

SCHMID, P., SELAK, S., KELLER, M., LUHAN, B., MAGYARICS, Z., SEIDEL, S., SCHLICK, P., REINISCH, C., LINGNAU, K., NAGY, E. & GRUBECK-LOEBENSTEIN, B. 2011. Th17/Th1 biased immunity to the pneumococcal proteins PcsB, StkP and PsaA in adults of different age. *Vaccine,* 29**,** 3982-3989.

SCHRODER, N. W. J., MORATH, S., ALEXANDER, C., HAMANN, L., HARTUNG, T., ZAHRINGER, U., GOBEL, U. B., WEBER, J. R. & SCHUMANN, R. R. 2003. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and Staphylococcus aureus activates immune cells via toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *Journal of Biological Chemistry,* 278**,** 15587-15594.

SENKOVICH, O., COOK, W. J., MIRZA, S., HOLLINGSHEAD, S. K., PROTASEVICH, I. I., BRILES, D. E. & CHATTOPADHYAY, D. 2007. Structure of a complex of human lactoferrin N-lobe with pneumococcal surface protein A provides insight into microbial defense mechanism. *Journal of Molecular Biology,* 370**,** 701-713.

SEPAKO, E., GLENNIE, S. J., JAMBO, K. C., MZINZA, D., IWAJOMO, O. H., BANDA, D., VAN OOSTERHOUT, J. J., WILLIAMS, N. A., GORDON, S. B. & HEYDERMAN, R.

S. 2014. Incomplete Recovery of Pneumococcal CD4 T Cell Immunity after Initiation of Antiretroviral Therapy in HIV-Infected Malawian Adults. *Plos One,* 9.

SHALLCROSS, L. J. & DAVIES, S. C. 2014. The World Health Assembly resolution on antimicrobial resistance. *J Antimicrob Chemother,* 69**,** 2883-5.

SHAPER, M., HOLLINGSHEAD, S. K., BENJAMIN, W. H. & BRILES, D. E. 2004. PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin. *Infection and Immunity,* 72**,** 5031-5040.

SIEVERS, F., WILM, A., DINEEN, D., GIBSON, T. J., KARPLUS, K., LI, W. Z., LOPEZ, R., MCWILLIAM, H., REMMERT, M., SODING, J., THOMPSON, J. D. & HIGGINS, D. G. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology,* 7.

SIMELL, B., LAHDENKARI, M., REUNANEN, A., KAYHTY, H. & VAKEVAINEN, M. 2008. Effects of ageing and gender on naturally acquired antibodies to pneumococcal capsular polysaccharides and virulence-associated proteins. *Clinical and Vaccine Immunology,* 15**,** 1391-1397.

SMIT, P., OBERHOLZER, D., HAYDEN-SMITH, S., KOORNHOF, H. J. & HILLEMAN, M. R. 1977. Protective efficacy of pneumococcal polysaccharide vaccines. *Jama,* 238**,** 2613-6.

SMITH, B. L. & HOSTETTER, M. K. 2000. C3 as substrate for adhesion of *Streptococcus pneumoniae*. *J Infect Dis,* 182**,** 497-508.

SONG, J. Y., NAHM, M. H. & MOSELEY, M. A. 2013. Clinical Implications of Pneumococcal Serotypes: Invasive Disease Potential, Clinical Presentations, and Antibiotic Resistance. *Journal of Korean Medical Science,* 28**,** 4-15.

STANDISH, A. J. & WEISER, J. N. 2009. Human neutrophils kill *Streptococcus pneumoniae* via serine proteases. *J Immunol,* 183**,** 2602-9.

STEINFORT, C., WILSON, R., RUTMAN, A., SYKES, D., TODD, H., WALKER, J., MITCHELL, T., SAUNDERS, K., ANDREW, P., BOULNOIS, G. & COLE, P. 1989. Pneumolysin produced by *Streptococcus pneumoniae* damages human respiratory epithelium in vitro. *Chest,* 95**,** S221-S221.

STOCKMANN, C., AMPOFO, K., PAVIA, A. T., BLASCHKE, A. J., MASON, E. O., PRESSON, A. P., FORNEY, L. J. & BYINGTON, C. L. 2016. Clinical and Epidemiological Evidence of the Red Queen Hypothesis in Pneumococcal Serotype Dynamics. *Clinical Infectious Diseases,* 63**,** 619-626.

STUDIER, F. W. & MOFFATT, B. A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology,* 189**,** 113-130.

TALBOT, U. M., PATON, A. W. & PATON, J. C. 1996. Uptake of *Streptococcus pneumoniae* by respiratory epithelial cells. *Infection and Immunity,* 64**,** 3772-3777.

TALKINGTON, D. F., CRIMMINS, D. L., VOELLINGER, D. C., YOTHER, J. & BRILES, D. E. 1991. A 43 kilodalton pneumococcal surface protein, PspA - isolation, protective abilities, and structural-analysis of the amino-terminal sequence. *Infection and Immunity,* 59**,** 1285-1289.

TARRAGO, D., FENOLL, A., SANCHEZ-TATAY, D., ARROYO, L. A., MUNOZ-ALMAGRO, C., ESTEVA, C., HAUSDORFF, W. P., CASAL, J. & OBANDO, I. 2008. Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. *Clinical Microbiology and Infection,* 14**,** 828-834.

TART, R. C., MCDANIEL, L. S., RALPH, B. A. & BRILES, D. E. 1996. Truncated *Streptococcus pneumoniae* PspA molecules elicit cross-protective immunity against pneumococcal challenge in mice. *Journal of Infectious Diseases,* 173**,** 380-386.

TOMLINSON, G., CHIMALAPATI, S., POLLARD, T., LAPP, T., COHEN, J., CAMBERLEIN, E., STAFFORD, S., PERISELNERIS, J., ALDRIDGE, C., VOLLMER, W., PICARD, C., CASANOVA, J. L., NOURSADEGHI, M. & BROWN, J. 2014. TLR-mediated inflammatory responses to *Streptococcus pneumoniae* are highly dependent on surface expression of bacterial lipoproteins. *J Immunol,* 193**,** 3736-45.

TORGERSON, T. R. & OCHS, H. D. 2007. Regulatory T cells in primary immunodeficiency diseases. *Curr Opin Allergy Clin Immunol,* 7**,** 515-21.

TORRES, A., BLASI, F., DARTOIS, N. & AKOVA, M. 2015. Which individuals are at increased risk of pneumococcal disease and why? Impact of COPD, asthma, smoking, diabetes,

and/or chronic heart disease on community-acquired pneumonia and invasive pneumococcal disease. *Thorax,* 70**,** 984-989.
TRAVIS, S. M., SINGH, P. K. & WELSH, M. J. 2001. Antimicrobial peptides and proteins in the innate defense of the airway surface. *Current Opinion in Immunology,* 13**,** 89-95.
TRZCIŃSKI , K., THOMPSON, C., MALLEY, R. & LIPSITCH, M. 2005. Antibodies to conserved pneumococcal antigens correlate with, but are not required for, protection against pneumococcal colonization induced by prior exposure in a mouse model. *Infection and Immunity,* 73**,** 7043-7046.
TRZCIŃSKI , K., THOMPSON, C. M., SRIVASTAVA, A., BASSET, A., MALLEY, R. & LIPSITCH, M. 2008. Protection against nasopharyngeal colonization by *Streptococcus pneumoniae* antigen-specific CD4(+) T cells. *Infection and Immunity,* 76**,** 2678-2684.
TU, A. H. T., FULGHAM, R. L., MCCRORY, M. A., BRILES, D. E. & SZALAI, A. J. 1999. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. *Infection and Immunity,* 67**,** 4720-4724.
URAKI, R., PIAO, Z., AKEDA, Y., IWATSUKI-HORIMOTO, K., KISO, M., OZAWA, M., OISHI, K. & KAWAOKA, Y. 2015. A Bivalent Vaccine Based on a PB2-Knockout Influenza Virus Protects Mice From Secondary Pneumococcal Pneumonia. *The Journal of infectious diseases,* 212**,** 1939-48.
VADESILHO, C. F. M., FERREIRA, D. M., GORDON, S. B., BRILES, D. E., MORENO, A. T., OLIVEIRA, M. L. S., HO, P. L. & MIYAJI, E. N. 2014. Mapping of Epitopes Recognized by Antibodies Induced by Immunization of Mice with PspA and PspC. *Clinical and Vaccine Immunology,* 21**,** 940-948.
VAN DE VEERDONK, F. L., GRESNIGT, M. S., KULLBERG, B. J., VAN DER MEER, J. W. M., JOOSTEN, L. A. B. & NETEA, M. G. 2009. Th17 responses and host defense against microorganisms: an overview. *Bmb Reports,* 42**,** 776-787.
VAN ROSSUM, A. M. C., LYSENKO, E. S. & WEISER, J. N. 2005. Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. *Infection and Immunity,* 73**,** 7718-7726.
VIDARSSON, G., DEKKERS, G. & RISPENS, T. 2014. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol,* 5**,** 520.
VINTINI, E. O. & MEDINA, M. S. 2011. Host immunity in the protective response to nasal immunization with a pneumococcal antigen associated to live and heat-killed Lactobacillus casei. *Bmc Immunology,* 12.
VITA, R., ZAREBSKI, L., GREENBAUM, J. A., EMAMI, H., HOOF, I., SALIMI, N., DAMLE, R., SETTE, A. & PETERS, B. 2010. The immune epitope database 2.0. *Nucleic Acids Res,* 38**,** D854-62.
WALPORT, M. J. 2001a. Advances in immunology: Complement (First of two parts). *New England Journal of Medicine,* 344**,** 1058-1066.
WALPORT, M. J. 2001b. Advances in immunology: Complement (Second of two parts). *New England Journal of Medicine,* 344**,** 1140-1144.
WANG, J. H., BARKE, R. A., CHARBONEAU, R., SCHWENDENER, R. & ROY, S. 2008. Morphine induces defects in early response of alveolar macrophages to *Streptococcus pneumoniae* by modulating TLR9-NF-kappa B signaling. *Journal of Immunology,* 180**,** 3594-3600.
WANG, S., LI, Y., SCARPELLINI, G., KONG, W., SHI, H., BAEK, C.-H., GUNN, B., WANDA, S.-Y., ROLAND, K. L., ZHANG, X., SENECHAL-WILLIS, P. & CURTISS, R., III 2010. Salmonella Vaccine Vectors Displaying Delayed Antigen Synthesis In Vivo To Enhance Immunogenicity. *Infection and Immunity,* 78**,** 3969-3980.
WANG, Y., JIANG, B., GUO, Y., LI, W., TIAN, Y., SONNENBERG, G. F., WEISER, J. N., NI, X. & SHEN, H. 2017. Cross-protective mucosal immunity mediated by memory Th17 cells against *Streptococcus pneumoniae* lung infection. *Mucosal Immunol,* 10**,** 250-259.
WARE, D., JIANG, Y., LIN, W. & SWIATLO, E. 2006. Involvement of potD in *Streptococcus pneumoniae* polyamine transport and pathogenesis. *Infection and Immunity,* 74**,** 352-361.
WARTHA, F., BEITER, K., ALBIGER, B., FERNEBRO, J., ZYCHLINSKY, A., NORMARK, S. & HENRIQUES-NORMARK, B. 2007. Capsule and D-alanylated lipoteichoic acids protect *Streptococcus pneumoniae* against neutrophil extracellular traps. *Cell Microbiol,* 9**,** 1162-71.

WATERA, C., NAKIYINGI, J., MIIRO, G., MUWONGE, R., WHITWORTH, J. A. G., GILKS, C. F. & FRENCH, N. 2004. 23-Valent pneumococcal polysaccharide vaccine in HIV-infected Ugandan adults: 6-year follow-up of a clinical trial cohort. *Aids,* 18**,** 1210-1213.

WEBER, J. R. & TUOMANEN, E. I. 2007. Cellular damage in bacterial meningitis: An interplay of bacterial and host driven toxicity. *Journal of Neuroimmunology,* 184**,** 45-52.

WEISER, J. N., BAE, D., FASCHING, C., SCAMURRA, R. W., RATNER, A. J. & JANOFF, E. N. 2003. Antibody-enhanced pneumococcal adherence requires IgA1 protease. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 4215-4220.

WEST, A. P., BRODSKY, I. E., RAHNER, C., WOO, D. K., ERDJUMENT-BROMAGE, H., TEMPST, P., WALSH, M. C., CHOI, Y., SHADEL, G. S. & GHOSH, S. 2011. TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature,* 472**,** 476-80.

WHO, 2016. Pneumonia. [Online] Available at: <http://who.int/mediacentre/factsheets/fs331/en/> [Accessed December 2016].

WOLTER, N., TEMPIA, S., COHEN, C., MADHI, S. A., VENTER, M., MOYES, J., WALAZA, S., MALOPE-KGOKONG, B., GROOME, M., DU PLESSIS, M., MAGOMANI, V., PRETORIUS, M., HELLFERSCEE, O., DAWOOD, H., KAHN, K., VARIAVA, E., KLUGMAN, K. P. & VON GOTTBERG, A. 2014. High Nasopharyngeal Pneumococcal Density, Increased by Viral Coinfection, Is Associated With Invasive Pneumococcal Pneumonia. *Journal of Infectious Diseases,* 210**,** 1649-1657.

WRIGHT, A., MORGAN, W. P., COLEBROOK, L. & DODGSON, R. W. 1914. Observations on prophylactic inoculation against pneumococcus infections. and on the results which have been achieved by it. *The Lancet,* 183**,** 87-95.

WRIGHT, A. K. A., BANGERT, M., GRITZFELD, J. F., FERREIRA, D. M., JAMBO, K. C., WRIGHT, A. D., COLLINS, A. M. & GORDON, S. B. 2013. Experimental Human Pneumococcal Carriage Augments IL-17A-dependent T-cell Defence of the Lung. *Plos Pathogens,* 9.

WRIGHT, A. K. A., FERREIRA, D. M., GRITZFELD, J. F., WRIGHT, A. D., ARMITAGE, K., JAMBO, K. C., BATE, E., EL BATRAWY, S., COLLINS, A. & GORDON, S. B. 2012. Human Nasal Challenge with *Streptococcus pneumoniae* Is Immunising in the Absence of Carriage. *Plos Pathogens,* 8.

WU, H. Y., NAHM, M. H., GUO, Y., RUSSELL, M. W. & BRILES, D. E. 1997. Intranasal immunization of mice with PspA (Pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. *Journal of Infectious Diseases,* 175**,** 839-846.

WYRES, K. L., LAMBERTSEN, L. M., CROUCHER, N. J., MCGEE, L., VON GOTTBERG, A., LINARES, J., JACOBS, M. R., KRISTINSSON, K. G., BEALL, B. W., KLUGMAN, K. P., PARKHILL, J., HAKENBECK, R., BENTLEY, S. D. & BRUEGGEMANN, A. B. 2013. Pneumococcal Capsular Switching: A Historical Perspective. *Journal of Infectious Diseases,* 207**,** 439-449.

XIN, W., LI, Y., MO, H., ROLAND, K. L. & CURTISS, R., III 2009. PspA Family Fusion Proteins Delivered by Attenuated Salmonella enterica Serovar Typhimurium Extend and Enhance Protection against *Streptococcus pneumoniae*. *Infection and Immunity,* 77**,** 4518-4528.

YAMAGUCHI, M., TERAO, Y., MORI, Y., HAMADA, S. & KAWABATA, S. 2008. PfbA, a Novel Plasmin- and Fibronectin-binding Protein of *Streptococcus pneumoniae*, Contributes to Fibronectin-dependent Adhesion and Antiphagocytosis. *Journal of Biological Chemistry,* 283**,** 36272-36279.

YOSHIMURA, A., LIEN, E., INGALLS, R. R., TUOMANEN, E., DZIARSKI, R. & GOLENBOCK, D. 1999. Cutting edge: Recognition of gram-positive bacterial cell wall components by the innate immune system occurs via toll-like receptor 2. *Journal of Immunology,* 163**,** 1-5.

YU, S. M., ZHANG, H., YAO, D., LIU, W., WANG, X. T., CHEN, X. T., WEI, Y. H., ZHANG, Z. H., WANG, J. N., YU, L. Q., SUN, H. N., WU, Z. J., YU, Y. Z., SONG, B. F., MA, J. Z., TONG, C. Y. & CUI, Y. D. 2015. Identification of CD4(+) T-cell epitopes on iron-regulated surface determinant B of Staphylococcus aureus. *Microbial Pathogenesis,* 89**,** 108-113.

YU, X., SPERLING, A., BLAIR, C., THOMPSON, K. & NACLERIO, R. 2004. Antigen stimulation of TH2 cells augments acute bacterial sinusitis in mice. *J Allergy Clin Immunol,* 114**,** 328-34.

YUSTE, J., SEN, A., TRUEDSSON, L., JONSSON, G., TAY, L. S., HYAMS, C., BAXENDALE, H. E., GOLDBLATT, F., BOTTO, M. & BROWN, J. S. 2008. Impaired opsonization with C3b and phagocytosis of *Streptococcus pneumoniae* in sera from subjects with defects in the classical complement pathway. *Infect Immun,* 76**,** 3761-70.

ZHANG, Q. B., LEONG, S. C., MCNAMARA, P. S., MUBARAK, A., MALLEY, R. & FINN, A. 2011. Characterisation of Regulatory T Cells in Nasal Associated Lymphoid Tissue in Children: Relationships with Pneumococcal Colonization. *Plos Pathogens,* 7.

ZHANG, Z., CLARKE, T. B. & WEISER, J. N. 2009. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *Journal of Clinical Investigation,* 119**,** 1899-1909.